doi: 10.30934/kusbed.915511 **e-ISSN:** 2149-8571

Kocaeli Üniversitesi Sağlık Bilimleri Dergisi

Özgün Araştırma / Original Article

http://dergipark.gov.tr/kusbed

POLY I:C-INDUCED TLR3 ACTIVATION ON OXIDATIVE STRESS LEVELS IN PC-3 (HORMONE-INSENSITIVE) AND LNCAP (HORMONE-SENSITIVE) AS PROSTATE CANCER CELLS

POLY I:C'NIN İNDÜKLEDİĞİ TLR3 AKTİVASYONUNUN PROSTAT KANSERİ HÜCRELERİ OLAN PC-3 (HORMONA DUYARSIZ) VE LNCAP'IN (HORMONA DUYARLI)OKSİDATİF STRES DÜZEYİNE ETKİLERİ

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Geliş Tarihi / Received: 13.04.2021 **Kabul Tarihi / Accepted:** 02.08.2021 **Yayım Tarihi / Published:** 21.03.2022

Abstract

Objective: We aimed to determine the role of Poly I:C-induced TLR3 activation on oxidative stress in two different prostate cancer cells [PC-3 (hormone insensitive) and LNCaP (hormone-sensitive)] for the first time. For this purpose, lipid peroxidation (MDA), hydrogen peroxide (H_2O_2) and proline amounts and superoxide dismutase (SOD) enzyme activity were examined.
Methods: The optimal concentration and time required for receptor stimulation with Poly I:C cells were determined by WST-1 analysis. Spectrophotometric methods determined biochemical parameters.
Results: The less cytotoxic concentration of 5 µM of Poly I:C on PC-3 and LNCaP cells was determined. A significant increase was observed in LNCaP cells in SOD activities after 6 and 24 hours. A significant increase in PC-3 and LNCaP cells' MDA levels was determined over 6 hours, while a significant decrease was observed in Poly I:C LNCaP after 24 hours. A significant increase in H_2O_2 concentration was detected in LNCaP cells, but a significant decrease was observed in PC-3 after 6 and 24 hours. The proline level showed a significant increase in LNCaP over 24 hours but not in the proline level in PC-3 cells after 6 and 24 hours.
Conclusion: The MDA, H_2O_2 and SOD activity levels were found to be significantly higher in hormone-sensitive LNCaP cells, while no significant changes were found in PC-3 cells treated with Poly I:C. Results were significantly different at the level of $p<0.05$ and $p<0.001$.

Keywords: *Cancer; proline, Poly I: C, Toll-like receptor 3.*

Öz

Amaç: Poly I:C ile indüklenen TLR3 aktivasyonunun iki farklı prostat kanseri hücresinde [PC-3 (hormona duyarsız) ve LNCaP (hormona duyarlı)] oksidatif stres üzerindeki rolünü ilk kez belirlemeyi amaçladık. Bu amaçla lipid peroksidasyonu (MDA), hidrojen peroksit (H_2O_2) ve prolin miktarlarına, süperoksit dismutaz (SOD) enzim aktivitesine bakılmıştır.
Yöntem: Reseptör uyarımı için gerekli olan ve hücre canlılığını destekleyen optimal Poly I:C doz ve süresi WST-1 analizi ile belirlendi. Biyokimyasal parametrelere spektrofotometrik yöntemler ile tayin edildi.
Bulgular: Poly I:C'nin PC-3 ve LNCaP hücreleri üzerinde daha az sitotoksik konsantrasyonunu olarak 5 µM belirlendi. SOD aktivitelerininde LNCaP hücrelerinde önemli bir artış 6 ve 24 saat sonra gözlenmedi. 6 saat boyunca PC-3 ve LNCaP hücrelerinin MDA seviyelerinde önemli bir artış belirlenirken, 24 saat sonra Poly I:C LNCaP hücrelerinde önemli bir düşüş gözlemlendi. LNCaP hücrelerininde H_2O_2 konsantrasyonunda önemli artış tespit edildi. Buna karşın 6 ve 24 saatlik Poly I:C uygulamalarından sonra PC-3 hücrelerinde H_2O_2 konsantrasyonunda önemli bir düşüş gözlendi. Prolin seviyesi LNCaP hücrelerinde 24 saat boyunca önemli bir artış gösterdi ancak PC-3 hücrelerinde hem 6 hem de 24 saat sonra prolin seviyesinde değişiklik olmadı.
Sonuç: Hormona duyarlı LNCaP hücrelerinde MDA, H_2O_2 ve SOD aktivite düzeyleri anlamlı olarak yüksek bulunurken Poly I:C ile tedavi edilen metastatik ve hormona duyarsız PC-3 hücrelerinde önemli bir değişiklik bulunmamıştır. İstatiksel veriler kontrol grubuyla karşılaştırıldığında $p<0,05$ ve $p<0,001$ düzeyinde anlamlı olarak farklıdır.

Anahtar Kelimeler: *Kanser, prolin, Poly I:C, Toll benzeri reseptör 3.*

Introduction

Toll-like receptor 3 (TLR-3) is a member of the TLR family and is expressed in many cells, including immune, endothelial and cancer cells.[1] TLR3 is found in the endosomes and initiates inflammatory pathways in response to pathogens.[2] The activation of TLR-3 by the dsRNA synthetic analogue Polyinosinic: polycytidylic acid (Poly I:C) has an antitumoral effect in cancer cells, including the prostate.[3] Several studies have shown that dsRNA (Double-stranded RNA) induced TLR-3 activation inhibits cell proliferation and induces apoptosis in many cancer cells.[4-6] Moreover, ROS has an important role in apoptosis induction.[7] Given the promising anticancer effects, using Poly I:C compounds as anticancer agents for chemo-immunotherapy warrants robust investigation.

Prostate cancer is one of the most frequently diagnosed tumours in men, and oxidative stress plays an important role in prostate carcinogenesis.[8-10]It can initiate DNA modifications in prostate tissue with oxidative stress.[11] Inflammation areas in prostate tissue generate free radicals and provide an important source of free radicals that induce pre-cancerous transformations.[12] Increasing the number of reactive oxygen species (ROS) causes damage to biological structures.[13] DNA damage causes changes in transcription and replication, activation of signal transduction pathways and genomic instability.[14] Cells are protected against harmful ROS activity through the antioxidant system [such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase].[15] In this context, oxidative stress plays an important role in developing prostate cancer.[16]

It is important to determine the roles of various signalling pathways at the molecular level and investigate their effects on prostate cancer oxidative stress markers. Therefore, in this study, we aimed to determine the role of Poly I:C-induced TLR3 activation on oxidative stress in two different types of prostate cancer cells [PC-3 (hormone-insensitive) and LNCaP (hormone-sensitive)] for the first time. For this purpose, lipid peroxidation (MDA), hydrogen peroxide (H_2O_2) and proline amount, SOD enzyme activity were determined as parameters. Thus, it aims to obtain information that will elucidate the relationship of the Poly I:C-induced TLR3 pathway with oxidative stress in cells in inflammation-related tumour formation such as prostate cancer.

Methods

Cell Culture Conditions

This study used PC-3 (hormone-insensitive) and LNCaP (hormone-sensitive) cells as prostate cancer cells. The cells were obtained from the American Type Culture Collection (ATCC) commercially. The cells were incubated in RPMI-1640 (Thermo Fisher) medium with 0.1% penicillin/streptomycin and 10 FBS% in 37°C 5% CO_2 incubator.

Cell Viability Assays and Poly I: C Treatment

The optimal concentration and time required for receptor stimulation with Poly I:C in PC-3 and LNCaP cells were determined by WST-1 and trypan blue exclusion (TBE) assay. For WST-1 assay, the cells were seeded in 96-well plates at $1x10^4$ cells/well and incubated at different times (6 and 24 hours) with different concentrations of Poly I:C (1, 5 and 10 μM). After incubation, the cells were incubated with WST-1 solution for 45 min. At the end of the incubation, the cells were analyzed with the optical reader at 450, and the viability of the negative control group without Poly I: C was considered 100%. For trypan blue exclusion assay, the cells were seeded in 96-well plates at $1x10^4$ cells/well and incubated at different times (6 and 24 hours) with different concentrations of Poly I:C (1, 5 and 10 μM). After incubation, cells were treated with trypsin/EDTA, and the cell suspension was collected and centrifuged. Cells were counted using a hemocytometer after the addition of trypan blue dye to the cell suspension. Poly I: C determined the dose and time for supporting the cell viability to TLR3 receptor stimulation. Cells were incubated in a growth medium without Poly I:C accepted as a negative control.

Oxidative Stress Analyses

Preparation of the cell lysates and determination of protein level

Cell lysates were isolated to determine the effects of Poly I:C on the total protein amount and oxidative stress parameters in PC-3 and LNCaP cells. For this purpose, the cells ($5x10^5$) were seeded in 6-well plates and treated with Poly I:C (5μM) for 6 and 24 hours. After treatment, cells were treated with RIPA cell lysis buffer (Sigma Aldrich) and centrifuged at 10.000 rpm for 10 minutes at 4°C. The supernatant obtained after centrifugation was used for further analysis. Additionally, the Bradford Assay method was used for the quantification of isolated proteins.[17] Bradford reagent (Bio-Rad, USA) was added to the protein samples and incubated for 5 minutes in the dark. After incubation, samples were measured at 595 nm with Nanodrop (Thermo Scientific, USA). Protein concentrations of samples were calculated by BSA standard curve. The proteins obtained after centrifugation was used for further analysis.

Determination of superoxide dismutase (SOD) activity

SOD activity was determined photochemically according to the method of Beauchamp and Fridovich.[18] SOD activity is determined by inhibiting the formation of NBT (nitrobluetetrazolium), formazan (blue-coloured crystals). Blue purple colour 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 amount of enzyme required to reduce 50% of the NBT kept under light at 560 nm.

Determination of malondialdehyde (MDA) content

Determination of MDA content among Poly I:C treatment groups was determined by the method of Ohkawa et al.[19] According to this method, the thiobarbituric acid test, which accepts MDA as the final product of lipid peroxidation, was used. The formation of malondialdehyde (MDA) content resulting from the thiobarbituric acid (TBA) reaction is accepted as a measure of lipid peroxidation. Results were calculated as MDA (nmol / g) in 1 ml of solution.[20]

Determination of proline content

The proline level among the Poly I:C treatment groups were determined according to the method determined by Bates et al.[21] Absorbance values were read in an ultraviolet spectrophotometer (Shimadzu UV mini-1240 spectrophotometer) at 520 nm wavelength. Proline

concentration was calculated as µmol / g by creating a proline standard curve.

Determination of hydrogen peroxide (H_2O_2) level

The H_2O2 level between the Poly I:C treatment groups were determined according to the method proposed by Jana and Choudhuri.[22] The absorbance at 410 nm of the last supernatant was measured, and the hydrogen peroxide concentration was calculated as µmol / g by creating a standard curve graph.

Statistical Analysis

Statistical analyzes were performed using the GraphPad 6.0 software package program. Differences between treatment groups depending on dose and time were determined by one-way analysis of variance (One-way ANOVA) post hoc analysis. In the analyzes, $p<0.001$ and $p<0.05$ were considered statistically significant.

Results

The Cell Viability Results

To determine the less cytotoxic concentration of Poly I:C, WST-1 analysis and TBE assay were conducted. The results showed that Poly I:C exerted a cytotoxic effect on PC-3 and LNCaP cells at 10 µM concentration and more prominent for 24 h ($p<0.001$), as shown in Figure 1. After 24 h of treatment with 5 µM Poly I:C, the growth rate of PC-3 and LNCaP cells reduced to 83.6% and 81.4%, respectively ($p<0.001$; Figure 1A and 1B). Additionally, the morphological images of the cells before and after being treated with Poly I:C were consistent with the WST-1 and TBE assay results, as shown in Figure 2. Therefore, 5 µM Poly I:C treatment was selected for further experiments due to the less cytotoxicity.

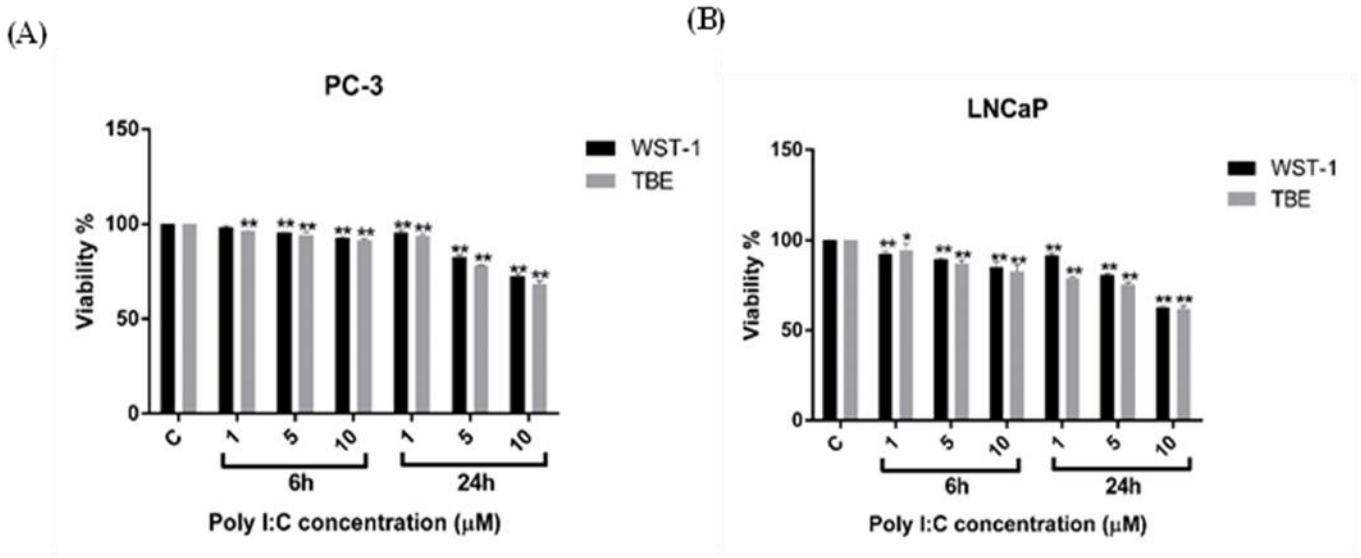

Fig 1. The viability of (A) PC-3 and (B) LNCaP cells as determined by WST-1 and TBE analysis after different concentrations of Poly I:C treatment (*$p<0.05$, **$p<0.001$).

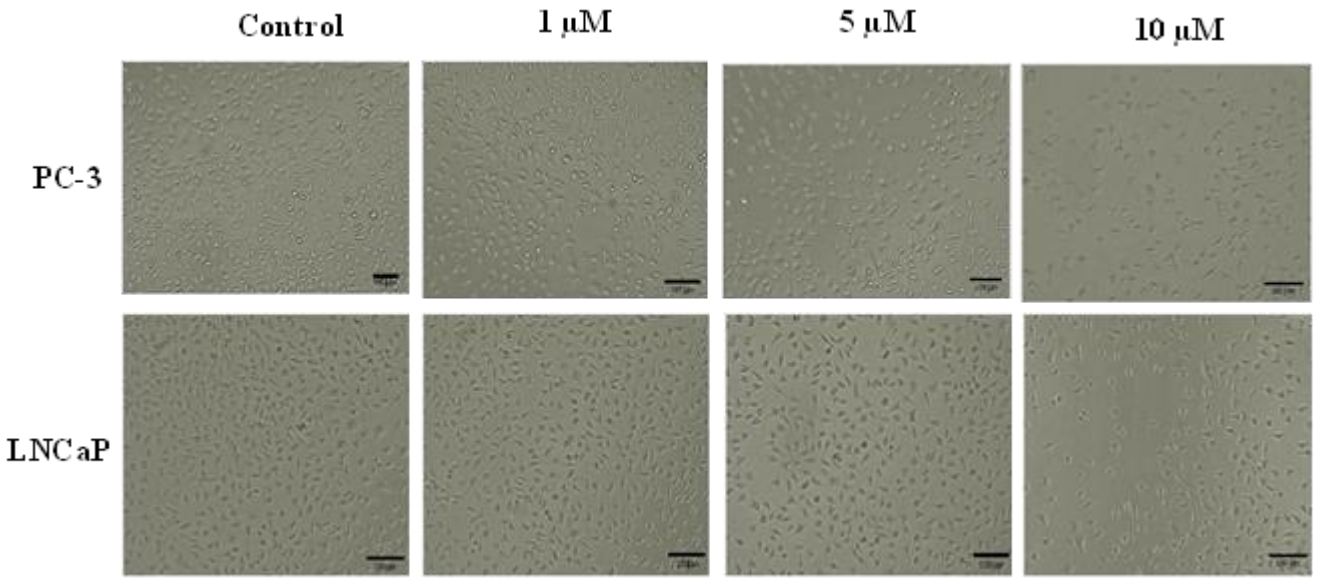

Fig 2. Cell morphology of PC-3 and LNCaP cells after being treated with 1, 5 and 10 µM Poly I:C for 24h. The Control group was not treated with Poly I:C in PC-3 and LNCaP cells.

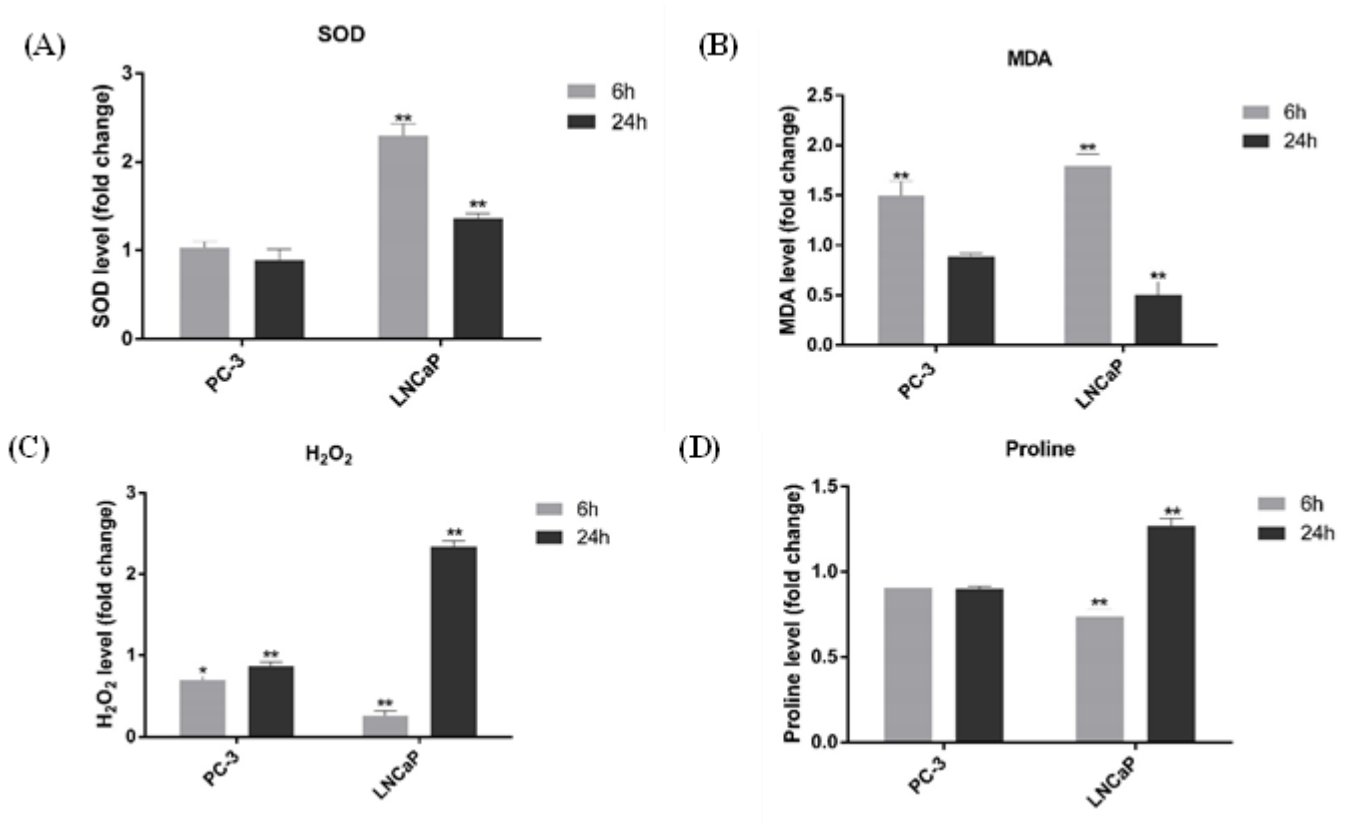

Fig 3. The effects of Poly I:C (5µM) induced TLR3 signalling in (A) SOD enzyme activity, (B) MDA, (C) H_2O_2 and (D) proline level in PC-3 and LNCaP cells for 6 and 24 h. The control group was not treated with Poly I:C was considered as the negative control, and the changes in the parameters were given as fold change, assuming the negative control group as "1". (SOD: superoxide dismutase, MDA: Malondialdehyde, H_2O_2: Hydrogen peroxide, *p<0.05, **p<0.001)

The Effect of Poly I: C on Oxidative Stress Parameters

The results of this study showed that the SOD activities of the cells incubated with Poly I:C for 6 and 24 h showed a significant increase 2.2- and 1.4-fold, respectively.in LNCaP cells compared to the control cells not treated with Poly I:C (1-fold). Nevertheless, no SOD activity change was observed in PC-3 cells treated with Poly I:C after both 6 and 24h (*p<0.05, **p<0.001, Figure 3A). A significant increase was observed in MDA levels of the PC-3 and LNCaP cells treated with Poly I:C (1.5- and 1.8-fold, respectively) for 6 h, but a significant decrease (0.5-fold) was observed in LNCaP cells after 24 h Poly I:C treatment (*p<0.05, **p<0.001, Figure 3B). Additionally, a similar significant increase was observed in H_2O_2 concentration (2.3-fold) of the LNCaP cells treated with Poly I:C for 24h, but a significant decrease was observed in PC-3 cells after both 6 and 24h Poly I:C treatments (*p<0.05, **p<0.001, Figure 3C). Besides, the proline level of the cells incubated with Poly I: C showed a significant increase (2.3-fold) in LNCaP cells for 24h but not in 6h (*p<0.05, **p<0.001, Figure 3D). However, no proline level change was observed in PC-3 cells treated with Poly I:C after both 6 and 24h (Figure 3D). Therefore, according to the results of this study, Total protein, MDA, H_2O_2, and SOD activity levels were found to be significantly higher in hormone-sensitive LNCaP cells. In contrast, no significant changes were found in the metastatic and hormone insensitive PC-3 cells treated with Poly I:C.

Discussion

In our previous study, Poly I:C induced TLR3 activation and expression have determined.[23] Depending on our findings, this study was aimed to determine the effect of the Poly I:C induced TLR3 activation on the oxidative status in prostate cancer cell lines (PC-3 and LNCaP), thereby the role of Poly I:C induced TLR3 activation on oxidative status of the prostate cancer cells. For this purpose, we analyzed the SOD activity and MDA, H_2O_2 and proline levels after treating the cells with Poly I:C as an agonist of TLR3.

Metalloenzyme Superoxide Dismutase (SOD) is one of the most effective intracellular enzymatic antioxidants. It is thought to act as the first defence mechanism against the harmful effects of reactive oxygen species. The SOD enzyme catalyzes the dismutation of the O_2^- molecule, allowing the formation of H_2O_2 and O_2, thus reducing the formation of OH- by removing the O_2^- molecule.[24] It has been stated that MnSOD functions as a tumour suppressor by modulating apoptotic and proliferation pathways *in situ*.[25,26] The malignant prostate epithelium has less MnSOD expression than benign prostate epithelium, and excess MnSOD expression in the prostate inhibits cancer cell growth *in vivo*.[27] Again, data indicating that many tumour types reduce MnSOD were reported by the same investigator.[27] Our study determined that the SOD activities of cells incubated with Poly I:C for 6 and 24 hours showed a significant increase (2.2 and 1.4-fold, respectively) in LNCaP cells compared to control cells not treated with Poly I: C (1-fold). This may be the effect of Poly I:C on enzyme expression. Especially the high increase observed in the 6 hours may be for the mechanism induced for apoptosis of the cancer cell. However, no changes in SOD activity were observed in PC-3 cells treated with Poly I:C after both 6 and 24 hours. We have analyzed the effect of the Poly I: C on the activity of SOD. In our findings, the decrease observed in antioxidant enzyme activity following poly(I:C) treatment

may describe the prooxidant effect of TLR3 seen on lipids and proteins in PC-3 cells.[28] Being an antioxidant, SOD may impact the prevention of prostate cancer.

Lipid peroxidation resulting from oxidative stress plays a role in various pathological conditions, including inflammation, atherosclerosis, neurodegenerative diseases, and cancer.[29] A study conducted on heterozygous mice stated that mice with the MnSOD mutation had a striking increase in mitochondrial lipid peroxidation, even though they lived to old age. The same study reported that the MnSOD mutation increased tumour formation.[30,31] MDA has an important role in the lipid peroxidation of polyunsaturated fatty acids, and cancer cells have an imbalance in redox status resulting in damage to DNA, protein and lipids.[32] It has been shown that lipid peroxidation products, malondialdehyde and 4-hydroxynonenal increase in colorectal cancer tissues, thyroid cancers, ependymal glial tumours and breast cancers.[33-35] The reasons for the increase in malondialdehyde and 4-hydroxynonenal levels in different tumours are due to the increase in enzymes that metabolize these products in tumour cells or the presence of inflammatory cells that can increase these factors.[29] These data show the importance of MDA amounts formed due to lipid peroxidation in tumour cells. In our findings, MDA levels were significantly higher in 6h than 24h treatment of Poly I:C in both PC-3 and LNCaP cells compared to the control. Polly I:C induced TLR3 activation can affect the MDA level, and it may be used as a non-invasive biomarker of oxidative stress, especially in hormone-dependent prostate cancer cells.

Reactive oxygen species (ROS) formation plays a critical role in the G2-M phase cell cycle. In a study by Xiao et al., treatment with diallyl trisulfide (DATS), a cancer chemopreventive agent derived from garlic, noted increased ROS production in human prostate cancer cells.[36] The produced ROS has been reported to cause a decrease in the protein level of cancer-associated Cdc25C and an increase in Ser216 phosphorylation. Besides, the fact that the Cdk inhibitor p21 causes an increase in the protein level is important data in the impaired cell cycle.[36] n another study, it was shown that exposure of HeLa cells to H_2O_2 destroyed the Cdc25C protein.[37] H_2O_2, which we have determined in our study, occurs because of the catalysis of superoxide in aerobic organisms by superoxide dismutase (SOD) enzyme, which has high catalytic activity. A similar significant increase in H_2O_2 concentration (2.3-fold) in LNCaP cells treated with Poly I:C for 24 hours in our study suggests that they are likely important signal mediators in cell cycle arrest. These results indicate that Poly I:C has a strong potential to investigate potential chemopreventive and therapeutic efficacy against prostate cancer in humans.

Proline plays an important role in cancer metabolism. Proline oxidase, which acts as the initiator of the proline cycle, is one of the rapidly and strongly induced genes by the tumour suppressor P53. Excessive proline oxidase expression delays the growth of xenograft tumours. Proline oxidase is significantly reduced in kidney tumours.[38] Proline oxidase responds to genotoxic stress and inflammatory and metabolic stress, mediating programmed cell death, on the other hand, survival by oppositely directed responses.[39] Although there is much to explain about the proline system, the data obtained about its role in cancer.[40-43] These data support the increase (2.3-fold) in proline level over 24 hours in LNCaP cells incubated with Poly I:C. However, no proline level changes were observed in PC-3 cells treated with Poly I:C after 6 and 24 hours.

Conclusion

The study has obtained results consistent with the literature that claimed that the prooxidant effect of TLR3 observed on lipids in PC-3 cells and TLR3 activation can affect the MDA level. These results demonstrated that Poly I:C should be seriously considered for further clinical research to determine the possible chemopreventive and therapeutic efficacy against prostate cancer in humans. These results also support the apoptotic role of TLR3 signalling by ROS induction in prostate cancer, especially in hormone-dependent cells, and TLR3 effects on oxidative stress in prostate cancer cells might contribute to the design of specific antagonists or therapies by targeting the TLR3 signalling in the treatment of prostate cancer.

Acknowledgement

The authors thank Dr Ozlem Aksoy from the Department of Biology at Kocaeli University for supporting our experiments.

Conflict of Interest

The authors have no conflicts of interest to disclose.

Financial Support

The authors declared that no financial support was received for this paper.

Author Contributions

ADO, BY: Design; ADO, BY: Analysis; BY: Literature search; ADO: Manuscript writing; ADO, BY: Critical review

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