E. Aydın Karataş and K.N. Bayındırlı / Hacettepe J. Biol. & Chem., 2022, 50 (1), 1-12

Research Article

Hacettepe Journal of Biology and Chemistry

journal homepage: www.hjbc.hacettepe.edu.tr

Enhanced Anticancer Potency of Gemcitabine in Combination With Propofol in Prostate Cancer

Prostat Kanserinde Gemsitabin ve Propofolün Birlikte Kullanımının Antikanser Potansiyeli

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ABSTRACT

In the present study, the effects of propofol which is an intravenous anesthetic on its own and simultaneously with gemcitabine were investigated on human prostate cancer brain metastasis DU145 and bone metastasis PC3 cells at both cellular and molecular levels. In the first stage of our study, toxic doses of these agents were determined by using the CVDK-8 and lactate dehydrogenase release test. In the following phases, TAC and TOS analyzes were performed to determine the biochemical effects of these agents on cell lines, and also western blot analysis was used to show the inhibition of important oncogenic PI3K/AKT/mTOR pathway in cells treated with these agents. Propofol was found to increase the effectiveness of gemcitabine in both cells. When propofol and gemcitabine were administered simultaneously at high concentrations, they reduced cell viability and increased LDH activity. According to the results obtained from the western blot analysis, the combination of these two agents was found to lead to synergistic inhibition of the PI3K/Akt/mTOR pathway.

Key Words

Cytotoxicity, gemcitabine, propofol, prostate cancer.

ÖZ

Mevcut çalışmada insan prostat kanseri beyin metastazı DU145 ve kemik metastazı PC3 hücreleri üzerine intravenöz bir anestezik olan propofolün, kemoterapötik bir ajan olan gemsitabin ile ayrı ayrı ve eş zamanlı olarak uygulanması durumunda oluşacak etkiler hem hücresel hem de moleküler düzeyde araştırıldı. Araştırmamızın ilk aşamasında hücre proliferasyon testi CVDK-8 ve laktat dehidrogenaz (LDH) salınım testi yardımıyla söz konusu ajanların toksik olan dozları belirlendi. Devam eden safhalarda ise bu ajanların hücre hatları üzerine olan biyokimyasal etkilerini tespit etmek için Toplam Antioksidan Kapasite (TAK) ve Toplam Oksidan Durumu (TOD) analizleri ve bu ajanlarla muamele edilen hücrelerdeki onkogenik proteinlerde ifade değişimlerini göstermek amacı ile de western blot analizi gerçekleştirildi. Hem DU145 hem de PC3 hücrelerinde propofolün gemsitabinin etkinliğini arttırdığı tespit edildi. Özellikle propofol ve gemsitabinin eş zamanlı olarak yüksek konsantrasyonlarda uygulandıklarında hücre canlılığını azalttığı ve LDH aktivitesini arttırdığı gözlemlendi. Western blot analizinden elde edilen sonuçlara göre, bu iki ajanın birlikte kullanımı PI3K/Akt/mTOR yolağının sinerjistik bir şekilde inhibe olmasına yol açtığı görüldü.

Anahtar Kelimeler

Sitotoksisite, gemsitabin, propofol, prostat kanseri.

Article History: Received: Sep 18, 2020; Revised: Jan 30, 2021; Accepted: May 22, 2021; Available Online: May 26, 2021.

DOI: : https://doi.org/10.15671/hjbc.796377

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INTRODUCTION

Globally, especially in developed countries, cancer of the prostate is one of the most widespread types of malignancies in men who are above 50 years old [1]. Every year, while an average of 1.6 million men are diagnosed with prostate cancer; about 366,000 men die from this disease [2]. Although there are several therapeutic approaches to treat prostate cancer including surgery, chemotherapy, radiation therapy, chemohormonal therapy, etc. [3, 4], there is no completely effective treatment method for prostate cancer [5]. The major disadvantage of chemotherapeutics are that they are toxic not only to the cancerous but also to the normal tissues [6]. This drawback could be eliminated by various chemotherapeutic combinations in which toxic substances are preferred in lower doses, and treatment potential is increased by additional utilization of a nontoxic substance with a distinct mode of action [7]. Therefore, combinations of chemotherapeutics with different active substances are considered as a new approach in the treatment of prostate cancer.

Many anesthetics are used in surgical oncology, in both before and after surgery [8, 9]. Propofol, one of these anesthetics, is a potent intravenous sedative and hypnotic drug [10]. Propofol, which is used in the treatment of cancer patients, has been shown to have anti-cancer properties in recent studies. Propofol, which is used in the treatment of cancer patients, has been found to have anticancer potential in addition to its anesthetic effect in recent studies. While some other anesthetics suppress immunity and assist cancer growth, propofol by acting in the opposite direction exhibits anticancer properties [11-17]. Sun and Sun [18] reported that propofol inhibited growth and metastasis by inactivating Raf1/ERK1/2 and Wnt/β-catenin pathways in hepatocellular carcinoma cells (Huh-7 and HepG2). Kang et al. [19] investigated the anticancer potential of propofol on Leydig cell cancer (MA-10 cell). According to the results obtained from this research, propofol induced apoptosis by activating Caspase and MAPK pathways, and inhibited the Akt pathway in MA-10 cells. In a study conducted on glioma cells, indicated that propofol repressed the migration and invasion of glioma cells by inhibiting the PI3K/AKT pathway [20]. In a recent study, it has been demonstrated that as concentration of propofol and treatment time increased, the cell viability of human cholangiocarcinoma (QBC939) cells decreased thus, the anti-cancer activity of propofol was showed on QBC939 cells [21].

Very few studies are available reporting the synergetic effects of gemcitabine in combination with propofol against cancer cells. However, there is no study on anticancer potential and associated mechanisms of combined gemcitabine and propofol against human prostate cancer brain metastasis DU145 and bone metastasis PC3 cells. In the present study, we examined the *in vitro* effects of combinational treatment of gemcitabine and propofol against DU145 and PC3 cells, as well as related mechanisms to ensure the scientific reason for clinical practice in the treatment of prostate cancer.

MATERIALS and METHODS

Cell Culture

DU145 human prostate cancer cell line derived from brain metastasis and PC3 human prostate cancer cell line derived from bone metastasis were used in the study. Both cell lines are androgen-independent prostate cancer cells and were cultured in sterile flasks (25 cm2) in RPMI1640 medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine at 37°C in 5% CO_2 incubator. Cells were passaged every 3 days considering the approximate growth rates of the cells.

Cell Viability Assay

DU145 and PC3 cells were plated in 96-well plates and incubated at 37°C for 24 hours to adhere to the bottom of the wells. After 24 hours, the medium in the wells was withdrawn. Cells were treated with different concentrations of gemcitabine (60, 30, 15, 7.5 and 3.75 µg/mL) and/or propofol (100, 75, 50, 25, 10, 5 and 1 µg/mL) in five replicates. The group treated neither with gemcitabine nor propofol served as the negative control and the group treated with the medium containing 20% DMSO served as the positive control. The effects of test substances on cell viability at the end of the optimum periods of incubation were investigated using the Cell Viability Detection Kit-8 (CVDK-8, EcoTech Biotechnology) following the manufacturer's protocol. Briefly, culture media of cells treated with test compounds were removed and medium containing 10% CVDK-8 solution was added to the wells. Changes in viability of cells were measured using the optical density values at 590 nm.

LDH Cytotoxicity Assay

The effects of gemcitabine and propofol on prostate cancer cells were also investigated using the lactate dehydrogenase release (LDH) test. DU145 and PC3 cells,

seeded into 96-well plates, were treated with gemcitabine and/or propofol. Cells treated with RPMI1640 complete medium served as a negative control group and cells treated with Triton-X served as the positive control group. The media of the cells that were incubated alone or in combination of gemcitabine and propofol were collected and transferred to other plates. The Pierce LDH Cytotoxicity Assay Kit commercially available from Thermo Scientific® was used in accordance with the manufacturer's instructions. Cell media to which the reaction solution was added was kept in the dark for 30 minutes. At the end of this period, absorbance values were obtained from the cell media treated with the termination solution at 490 and 680 nm using a spectrophotometer device.

Total Antioxidant Capacity Assay

Total Antioxidant Capacity (TAC) analysis was performed using the Total Antioxidant Status Assay Kit purchased from Rel Assay Diagnostics® to evaluate the oxidative stress in DU145 and PC3 cells treated with gemcitabine and/or propofol. 30 µl of medium was collected from the cells treated with gemcitabine and/or propofol and transferred to 96-well plates and manufacturer's protocol was followed to measure the TAC of cells at 660 nm. The outputs obtained were processed as specified in manufacturer's protocol to get the TAC levels.

Total Oxidant Status Assay

Total Oxidant Status (TOS) analysis was performed using Total Oxidant Status Assay Kit obtained from Rel Assay Diagnostics® to determine the differential oxidant status in cells treated with gemcitabine and/or propofol. 500 µl Reagent 1 solution was added to 75 µl medium collected from cells in 96-well plates and the first absorbance measurement was performed at 530 nm. Then, 25 µl Reagent 2 solution was added to all wells and after 10 minutes, the second absorbance measurement was carried out at 530 nm. The outputs obtained were processed as specified in manufacturer's protocol to get the TOS levels.

Western Blot Analysis

DU145 and PC3 cells, which were seeded in 6-well plates at 1.5x105 cells per well, were treated with propofol and/or gemcitabine. After treatment, cells were washed with ice cold PBS. Cell lysates were prepared using RIPA Lysis Buffer (EcoTech Biotechnology, Turkey) containing PMSF and protease inhibitor cocktail. Cell lysates were mixed with equal amounts of 2x Laemmli Sample Buffer and boiled at 100°C for 5 minutes. Equal amounts of proteins were electrophoresed with 10% SDS/PAGE and then transferred to PVDF membranes. Membranes were blocked with 5% non-fat dry milk (EcoTech Biotechnology, Turkey) in PBS-T buffer at room temperature. Blocked membranes were incubated at 4°C overnight with primary antibodies: pGSK3β (1:500, Santa Cruz Biotechnology®), β-actin (1:200, Santa Cruz Biotechnology®). After washed three times with PBS-T, membranes were probed with horseradish peroxidase (HRP)-linked secondary antibodies for 1h at room temperature. Proteins were visualized using ClearBand ECL Western Blotting Substrate (EcoTech Biotechnology, Turkey) following the manufacturer's instructions.

Statistical Analysis

Data were presented as the average of at least three replicates. Statistical differences were evaluated using Student's T-Test. Significant differences were accepted as * $p < 0.05$.

RESULTS

The cytotoxic effects of gemcitabine, propofol and their combination on both DU145 and PC3 cells were investigated using CVDK-8 and LDH tests. Our results demonstrated that gemcitabine decreased cell viability of DU145 cells at concentrations of 30 and 60 µg/ml, and propofol at concentrations of 50 and 100 µg/ml (Figure 1).

When administered together, propofol significantly decreased cell viability starting from concentration of 25 µg/ml. Besides, LDH experiments revealed that in DU145 cells treated with gemcitabine alone, LDH activity increased in all concentrations but the highest LDH activity was detected at 60 µg/ml. On the other hand, when propofol was administered alone, an increased LDH activity was observed at concentrations of 50 and 100 µg/ml. When both agents were applied together, propofol increased the LDH activity starting from the concentration of 25 µg/ml (Figure 2).

According to the cell proliferation analysis of PC3 cells, gemcitabine decreased the cell viability at concentrations of 15 and 30 µg/ml, while 75 and 100 µg/ml concentrations of propofol decreased viability of PC3 cells. A decrease in viability was observed in all treatment groups where both agents were administered together dependent on the concentration (Figure 3).

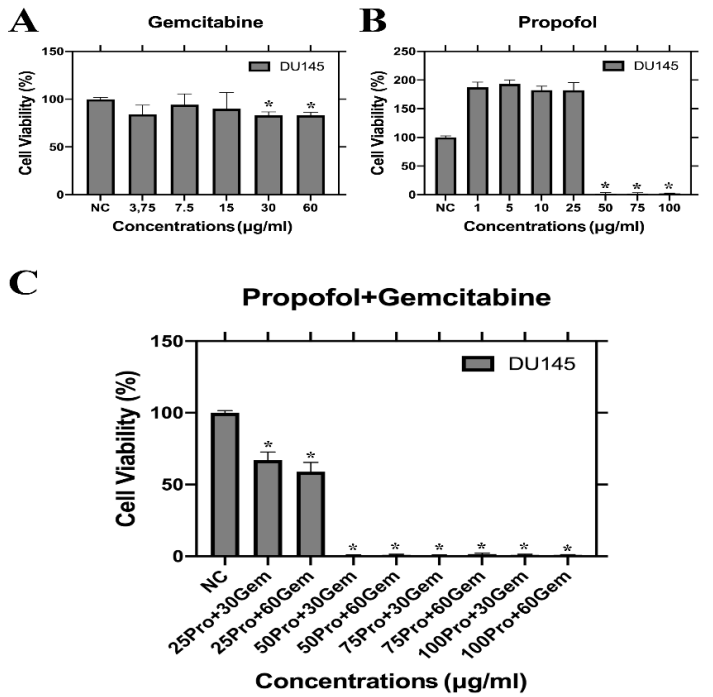

Figure 1. Relative cell viability in DU145 cells treated with gemcitabine and/or propofol.

In the meanwhile, a statistically significant increase was observed in the amount of LDH released into the extracellular environment at all gemcitabine concentrations and propofol concentrations of 75 and 100 µg/ml in PC3 cells. When PC3 cells were treated with both agents together, an increase in LDH activity was observed in all concentrations (Figure 4).

Oxidative stress responses created by gemcitabine and propofol in cells were determined by TAC and TOS methods. When concentrations of 30 and 60 µg/ml of gemcitabine and 25, 50, 75, and 100 µg/ml of propofol were applied to DU145 cells alone or in combination, there was no statistically significant change in TAC and TOS values (Figure 5-6).

On the other hand, while gemcitabine decreased the TAC values in both concentrations of gemcitabine in PC3 cells, propofol decreased TAS value at concentrations of 75 and 100 µg/ml. When 30 µg/ml concentration of gemcitabine was applied together with 50 µg/ml and above concentrations of propofol, the TAC value decreased significantly (Figure 7).

Besides, in PC3 cells, gemcitabine did not change TOS values at any applied concentrations, while propofol increased significantly the TOS values at concentrations of 50 µg/ml and above. When both were applied simultaneously, propofol increased the TOS value at concentrations of 50 µg/ml and above (Figure 8).

The phosphorylated (active) version of GSK3β, which is one of the important downstream effectors of PI3K/Akt/mTOR pathway that has been shown to be involved in prostate carcinogenesis, was evaluated by Western blot in order to examine the potential mechanism of actions of gemcitabine and propofol on prostate cancer cells. pGSK3β was significantly decreased in gemcitabine treated DU145 and PC3 cells compared to the corresponding controls. Propofol, on the other hand, did not cause a significant change in the amount of pGSK3β in DU145 cells, however, caused a decrease in PC3 cells. This can be interpreted as both gemcitabine and propofol interfere with the viability of prostate cancer cells by inhibiting the PI3K/Akt/mTOR pathway. More importantly, the level of pGSK3β in DU145 and PC3 cells treated with gemcitabine and propofol together was much more strongly inhibited than when these agents were administered alone. The combination of these two agents led to synergistic inhibition of the PI3K/Akt/mTOR pathway (Figure 9).

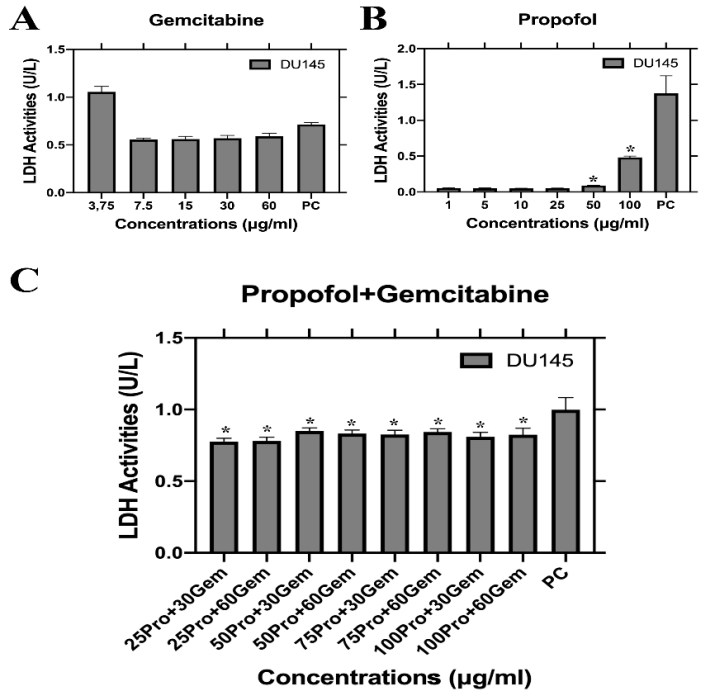

Figure 2. Relative LDH activity in DU145 cells treated with gemcitabine and/or propofol.

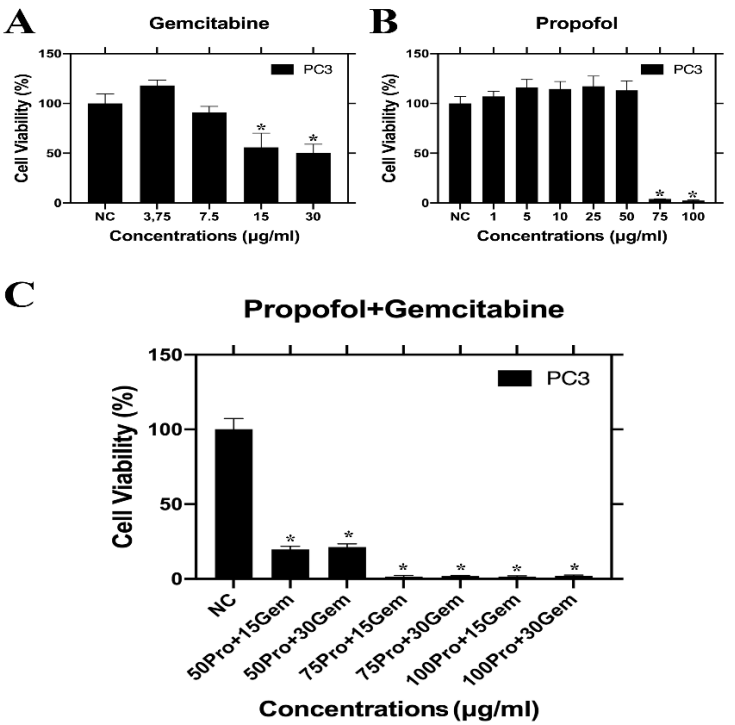

Figure 3. Relative cell viability in PC3 cells treated with gemcitabine and/or propofol.

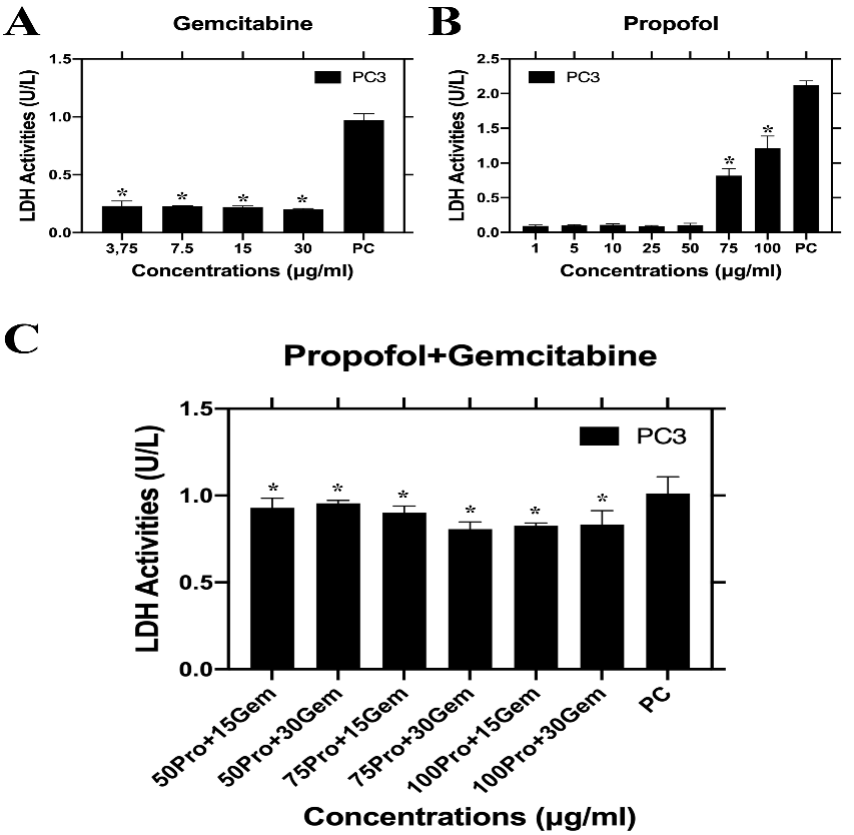

Figure 4. Relative LDH activity in PC3 cells treated with gemcitabine and/or propofol.

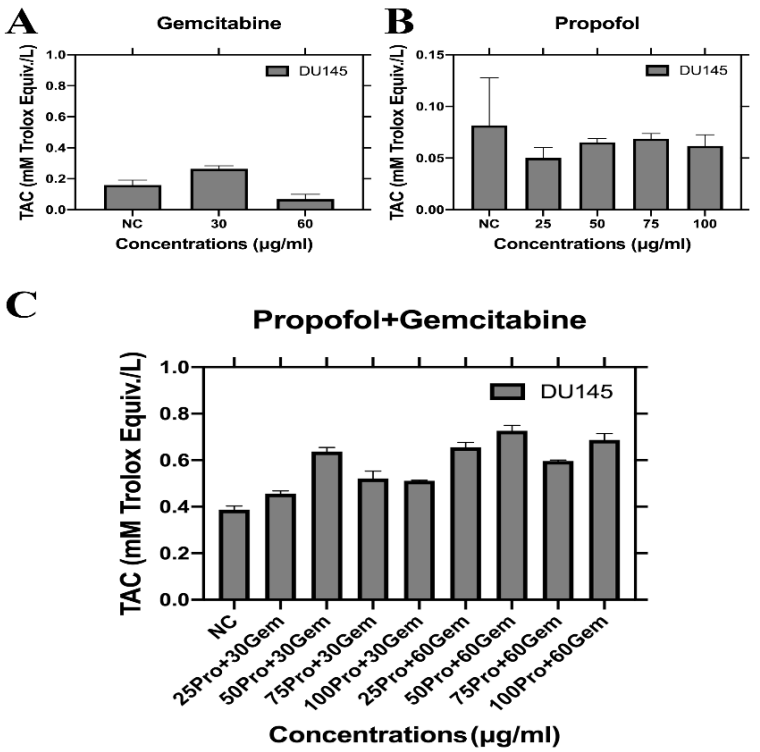

Figure 5. Relative TAC values in DU145 cells treated with gemcitabine and/or propofol.

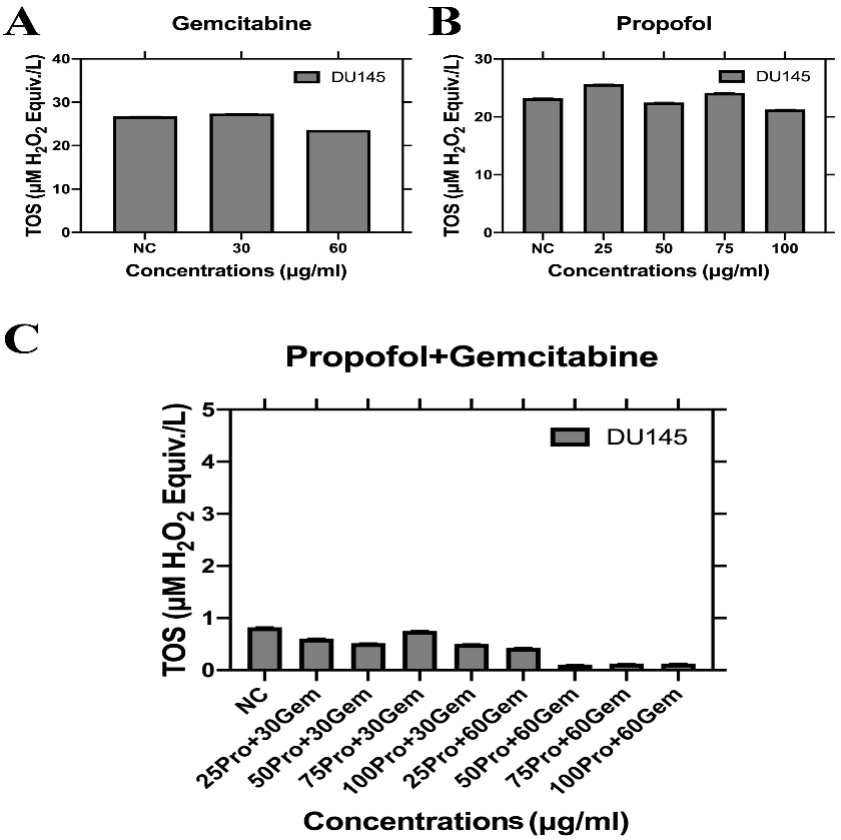

Figure 6. Relative TOS values in DU145 cells treated with gemcitabine and/or propofol.

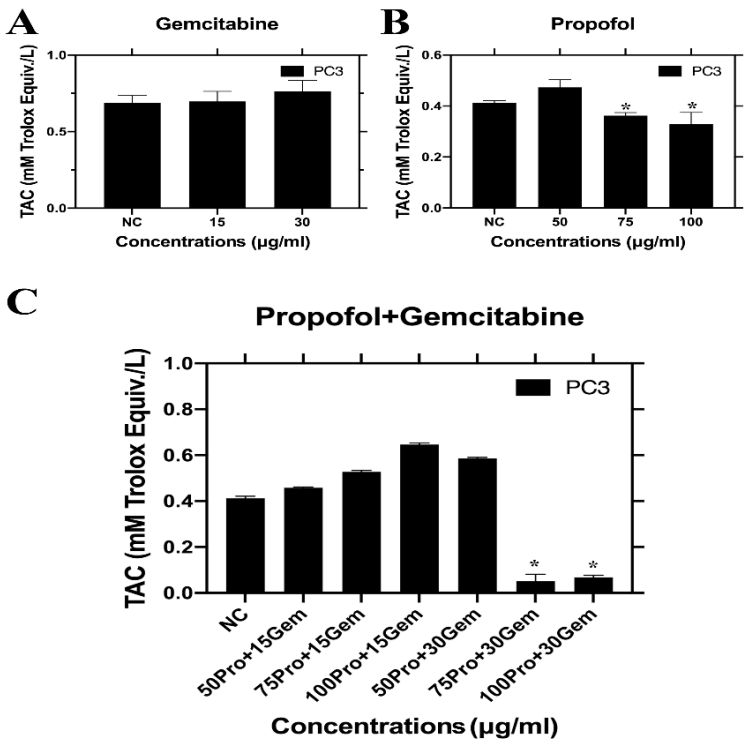

Figure 7. Relative TAC values in PC3 cells treated with gemcitabine and/or propofol.

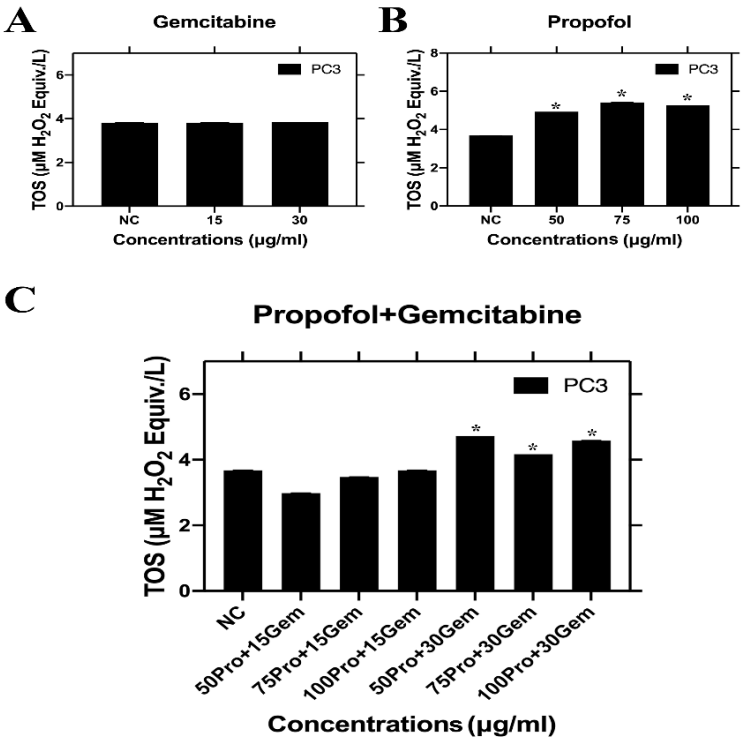

Figure 8. Relative TOS values in PC3 cells treated with gemcitabine and/or propofol.

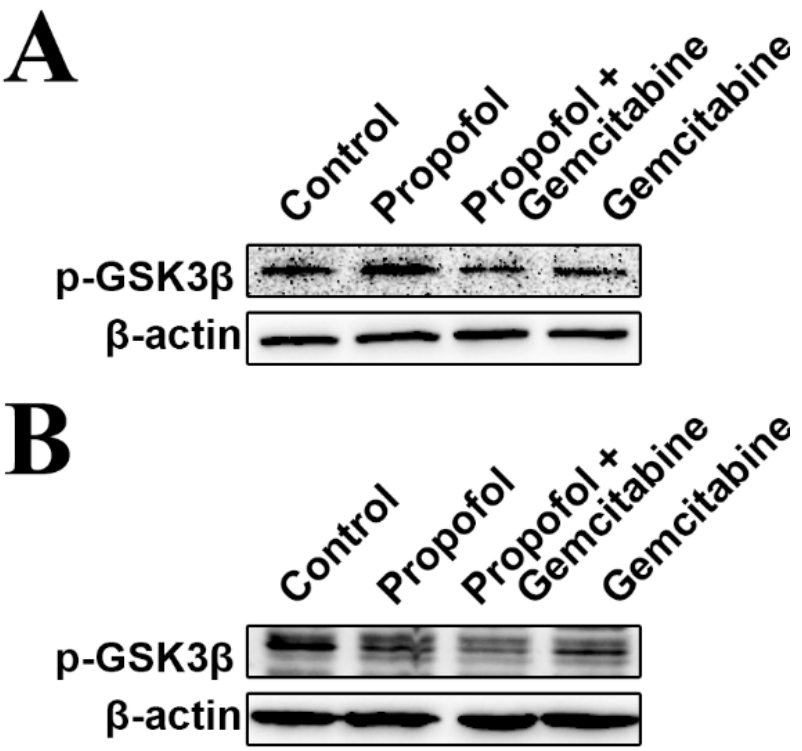

Figure 9. Relative pGSK3β levels in A) Du145 and B) PC3 cells treated with gemcitabine and/or propofol.

DISCUSSION

Prostate cancer, the second most common type of cancer in men, is one of the most important causes of death in men worldwide [22-24]. Treatment options of prostate cancer are quite diverse including surgery, chemotherapy, radiotherapy, hormone therapy and target therapy [25-28], however, chemotherapy is the most commonly used therapeutic strategy [29]. The biggest disadvantage of chemotherapeutics or chemotherapy damages not only cancerous cells both also healthy cells and tissues [30]. In recent years, chemotherapeutic combinations that minimize this disadvantage are considered as a new treatment approach.

In cancer patients, several anesthetics agents are used in both before and after surgery periods. But, detailed effects of aforementioned anesthetics on cancer cells are not yet known [8]. Propofol, which is an intravenous anesthetic, is used especially to relieve the pain of operated patients [31, 32]. In recent years, propofol has been reported to have anticancer activity on different cells including glioma [32], squamous cell carcinoma [33], and breast cancer cells [34].

In this present study, we investigated the use of propofol in combination with gemcitabine, which is a widely used valuable chemotherapy drug, and evaluated its potential against prostate cancer PC3 and DU145 cell lines. This experimental study revealed that the chemo-sensitization of cancer cells by propofol was succeeded via increase in gemcitabine-induced cell death as indicated by more marked decrease in cell viability compared with single gemcitabine treatment. We showed that adjuvant propofol treatment critically increased tumor cell death compared with both agents alone. While gemcitabine (30 and 60 µg/ml doses) caused approximately 17% cell death, 50 and 100 µg/ml doses of propofol caused approximately 98% cell death in DU145 cells. Besides, 25 µg/ml dose of propofol were non-toxic in DU145 cells. The observation is remarkable because, when gemcitabine (30 and 60 µg/ml doses) and 25 µg/ml doses of propofol were administered simultaneously, the cell mortality rate reached approximately 42%. Similarly, while gemcitabine (15 and 30 µg/ml doses) caused approximately 50% cell death, 75 and 100 µg/ml doses of propofol caused approximately 97% cell death in PC3 cells. Besides, 50 µg/ml dose of propofol were non-toxic in PC3 cells. The observation is similarly remarkable since, when gemcitabine (15 and 30 µg/ml doses) and 50 µg/ml doses of propofol were administered simultaneously, the cell mortality rate reached approximately 50%. As can be seen from these results, propofol increased cell death in both cell lines. In parallel with the previously obtained proliferation-reducing effect of propofol, we indicated that propofol alone not only importantly inhibited prostate cancer DU145 and PC3 cell proliferation, but also increased gemcitabine-induced cell death as observed by CVDK-8 and LDH assays. Just like our results, Xu et al. [35] found that low concentrations of propofol increased the cell viability; however, high concentrations of propofol decreased the viability of cells on rat hippocampal neuron culture. Du et al. [7] investigated the effect of propofol on cell viability alone and in combination with gemcitabine by MTT assay on pancreatic cancer MIA-PaCa-2 cells. While propofol inhibited cell proliferation, depending on dose and time, application of gemcitabine in combination with propofol decreased cell viability more effectively compared to both propofol and gemcitabine alone. Studies in the human esophageal squamous cell carcinomas (ESCC) EC-1 cells in vitro have provided evidence supporting a dose-dependent inhibition of propofol on cell proliferation [33]. Xu et al. [32] reported that propofol exposure dose-dependently inhibited the cell viability of the glioma cells (LN299) by using CCK-8 assay. The study in murine melanoma B16F10 cells also showed that propofol significantly inhibited melanoma cell growth in response to a dose- and time- dependent manner compared to control [36]. Previous researches have reported that propofol caused decrease in cell proliferation on non-small cell lung cancer cells [37], glioblastoma cells and astrocytes [38].

Oxidative stress, which is a situation that occurs when the balance between the production and destruction of free radicals and reactive oxygen species is disrupted, is one of the major players in the pathogenesis including initiation, progression, invasion and metastasis of cancer [39-41]. When a healthy cell transforms into a cancer cell, the amount of ROS increases, thus oxidative stress increases. However, ironically, it is very frequently preferred to trigger death by increasing ROS production in cells in almost all non-surgical cancer treatment methods including chemotherapy, radiotherapy and photodynamic therapy [42]. As a matter of fact, we determined that propofol increased TOC levels in concentrations above 50 µg/ml, however when applied together with gemcitabine, it increased TOS levels more effectively in PC3 cells. It was also observed that at the same doses, propofol decreased TAC levels in PC3 cells.

On the other hand, lactate dehydrogenase, which is an enzyme with two large subunits, causes oxidative stress leading to cell death [43]. As cell death increases, the level of LDH in the medium increases. The biochemical tests revealed that when applied simultaneously (with gemcitabine) propofol more effectively increased LDH enzyme activity compared to treatment of agents one by one in both cell lines. On a study conducted in PANC-1 pancreatic cancer cells was identified that dose-dependent propofol treatment increased LDH release. In the same study, it was also determined that the treatment of dose-dependent propofol reduced cell viability [44]. Liang and Dong [45] reported that the LDH activity increased with the treatment of propofol and this increase was considerable compared to the non-propofol treated control in human colon cancer cell line SW480.

PI3K-AKT-mTOR signaling pathway is aberrantly activated at a relatively high frequency in prostate cancer patients [46-48] and inhibitors of this pathway are currently under investigation as potential therapeutic agents against prostate cancer [46-52]. It has been found that gemcitabine does not change or even can activate the PI3K-AKT-mTOR signaling pathway in cancer cells through triggering production of ROS that weakens the anti-tumorigenic potential and therapeutic success of gemcitabine [53]. Here in this study, we demonstrated that administration of propofol in addition to gemcitabine to prostate cancer cells significantly inhibited the downstream effector of PI3K-AKT-mTOR signaling pathway, GSK3β, which increased the anti-proliferative potential of gemcitabine compared to its use alone.

Our study provides new insights into the anticancer effects of propofol on the behavior of two different prostate cancer cells (DU145 and PC3). The results of our present study indicated that propofol significantly decreased cell viability and caused an increase in some oxidative stress indicators in DU145 and PC3 cells. Besides, treatment of prostate cancer cells with propofol along with gemcitabine significantly inhibited PI3K-AKT-mTOR signaling pathway resulting in enhanced cell death exerted by gemcitabine. When all the results are taken together, there is evidence that propofol increased the effectiveness of a chemotherapeutic agent like gemcitabine. However, it should not be forgotten that these studies were carried out in vitro. Although propofol has these features in vitro conditions, it can exhibit very different features in vivo. Therefore, further studies, particularly in vivo, are needed to confirm the clinical relevance of propofol in cancer patients.

Acknowledgments

This study was financed by Erzurum Technical University Scientific Research Project (Grant Number: 2019/20).

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