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RESEARCH ARTICLE

EFFECTS OF CURCUMIN TREATMENT ON CELL ENERGY STATUS, LEVELS OF MITOCHONDRIAL ENZYMES, AND GENE EXPRESSION OF GLUCOSE-RELATED MECHANISM IN PANCREATIC CANCER CELL LINES

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ABSTRACT

Purpose: Curcumin is an active component of turmeric, has antitumor, immunomodulatory, anti-inflammatory effects. This study aimed to investigate the effects of the administration of curcumin on the energy metabolism, the abnormal redox defense mechanism profile, the malignant transformation indicator of Panc-1 and BxPC-3 pancreatic cancer cells.
Material and Methods: BxPC-3 and Panc-1 cells were treated with various concentrations of curcumin (20 μM, 40 μM, 50 μM, 60 μM, 80 μM, 100 μM, and 125 μM) for 24 h. Cell lysate Adenosine triphosphate (ATP), Adenosine diphosphate (ADP), Adenosine monophosphate (AMP), Manganese superoxide dismutase (MnSOD), and cytochrome p450 reductase (CPR) concentrations were analyzed with HPLC and ELISA methods. Genes expression of Lactate dehydrogenase (*LDH*), mitochondrially encoded ATP synthase membrane subunit 6 (*MTATP6*), Glucose transporter 1 (*GLUT1*), and cytochrome p450 were analyzed by real-time quantitative PCR.
Results: IC_{50} values for 24 hours were found as 47.26 μM in BxPC-3 and 45.84 μM in Panc-1 cells. Treatment with curcumin inhibited oxidative stress by increasing MnSOD enzyme levels. ATP levels did not change in BxPC-3 cells, but they increased in Panc-1 supplemented with curcumin. Gene expression of *GLUT-1* was significantly down regulated when using at 45 μM concentration of curcumin, which also affected glucose consumption in both cells.
Conclusion: Curcumin showed anti-proliferative and antioxidant effects.

Keywords: ATP, BxPC-3, curcumin, CPR, MnSOD, Panc-1

INTRODUCTION

Pancreatic cancer (PC) remains the fourth leading cause of cancer-related death with a 5-year survival rate and affects people less than 9% worldwide (1). PC is difficult to treat because of its heterogeneous nature, the microenvironment of the desmoplastic tumor, and the high rate of metastases (2). The use of bioactive compounds derived from fruits has

increased in recent years due to their potential health benefits and anticancer effects. Due to the biodiversity of bioactive compounds in fruits, they are used in the field of health and the treatment of pancreatic cancer (3, 4). This suggests that certain dietary factors may contribute to anti-cancer effects (5, 6).

Curcumin is the main active component of turmeric (7). Dinkova et al. (1999) found that the beta-diketone and the hydroxyl groups in the ortho-position on the aromatic rings in curcumin contribute to the regulation of phase 2 detoxification enzymes (7). Curcumin supplementation has been shown to reduce oxidative stress in certain diseases, such as cardiovascular diseases, diabetes, Alzheimer's disease, and multiple sclerosis (8, 9). Furthermore, it has been postulated that curcumin has features, such as anti-inflammatory (10), antitumor (11), immunomodulatory (12), renoprotective (13), hepatoprotective (14) hypoglycemic (15) and antimicrobial (16).

Oxidative stress is an important mechanism in the pathogenesis of many diseases, including cancer. Production of reactive oxygen species (ROS) and DNA damage is the first step in carcinogenesis induced by inflammatory processes (17). Functionally, cytochrome P450 (CYPs) has two important roles; those that catalyze endogenous substrate oxidation, such as hormones, and those that catalyze exogenous substrate oxidation, such as drugs, and other xenobiotics 18). The expression of drug-metabolizing CYP is largely localized in the liver and gastrointestinal tract, but an aberrant expression of specific isoforms of CYP has been identified in many solid tumors (18). Among CYPs, CYP3A4 is one of the potential therapeutic targets, in which it binds to a wide range of substrates and metabolizes more than 60% of the drugs (19, 20). CYP2E1 is composed of 7 % of CYP450 enzymes in the liver (21). CYP2E1 is a potent cellular pro-oxidant (22). Studies have shown that increased CYP2E1 level and activity are associated with increased ROS production and exacerbation of peroxide oxidation (23, 24). High expression of CYP3A4 was found in breast cancer cell lines (25).

Tumor cells frequently increase glucose consumption and lactate production even in the presence of physiological oxygen concentrations and functional mitochondria; this aerobic glycolysis is known as the Warburg effect (26). While normal cells obtain energy through the respiratory chain, cancer cells prefer the aerobic glycolysis pathway (27). In this process, there is an increase in Lactate dehydrogenase (LDH) activity which is a specific catalytic enzyme that catalyzes pyruvate to lactic acid in the presence of Nicotinamide adenine dinucleotide ($NADH+H^+$) as an electron donor (28). Thus, increased activity of this enzyme may serve as an indicator of malignant transformation (28). The decrease in mitochondrial membrane potential leads to an increase in ROS production. Both inadequate energy supply and increased oxidative stress contribute to the resulting pathological phenotype. The threshold for emergence of ATP synthase defect and subsequent metabolic remodeling is 30% of residual ATP synthase activity (reference). When the ATP synthase content falls below 30%, metabolic adaptations are sufficient to sustain proliferation under glucose-rich conditions (29). Glucose transporter 1 (*GLUT-1*) is the rate-limiting transporter for cell glucose uptake and is associated with anaerobic glycolysis reactions in cancer cells (30). *GLUT-1* expression increases in tumor cells due to the effect of hypoxia and decreased oxidative phosphorylation, as energy is required for proliferation (31-33).

Superoxide dismutase (SOD) is the most important antioxidant enzyme in aerobic cells and is responsible for scavenging superoxide radicals and protecting cells against DNA damage. SOD catalyzes the dismutation of hydrogen peroxide and molecular oxygen (34). As the primary antioxidant enzyme in mitochondria, MnSOD plays a key role in protecting cells against ROS by eliminating excessive superoxide radicals (35).

- Pancreatic cancer is an aggressive tumor types and has high mortality rate. Today, due to its insensitivity to the most treatment options, the researchers has been focused on the new strategies in the treatment of pancreatic cancer.

This study aimed to investigate the effects of curcumin treatment on the energy metabolism, the abnormal redox defense mechanism profile, and the malignant transformation in Panc-1 and BxPC-3 human pancreatic cancer cells. In the evaluation of energy metabolism, it is planned to determine the cell's ATP, ADP, and AMP levels and calculate the energy charge. Expression levels of *LDH* MTATP*6*, *GLUT-11*, and cytochrome p450 genes (*CYP2E1, CYP3A4*) were analyzed to determine the biochemical metabolic events of anaerobic glycolysis, the metabolic pathways that cancer cells use for energy, and the sign of detoxification, respectively. In addition, it also aimed to evaluate the effect of

curcumin application on MnSOD and CPR concentrations, which have an important role in maintaining cellular redox homeostasis and also the regulation of detoxification. Therefore, we aimed to clarify curcumin, a promising herbal agent in the treatment of cancer, according to the metabolic critical points of pancreatic cancer cells.

MATERIAL AND METHODS

This research was approved by Necmettin Erbakan University Ethics Committee (no: 2019/1666, decision date:18/01/2019). This study was supported by Necmettin Erbakan University Scientific Research Coordinator (project number:181215004). The present study aimed to investigate how the application of curcumin in various doses affects the values of MTATP6, MnSOD, CPR, LDH, GLUT-1, CYP2E1, CYP3A4, ATP, ADP, AMP in Panc-1 and BxPC-3 human pancreatic cancer cells. Curcumin IC_{50} values were also determined in this study.

Cell culture and cell vialibility (MTT assay)

Human PC cell lines (BxPC-3 and Panc-1) were purchased from the ATCC (Manassass, VA, USA), and cultured in RPMI-1640 (Gibco, UK) and DMEM (Gibco, UK) media supplemented with 10 % FBS (Gibco, USA) and 1 % penicillin/streptomycin. All of the cells were cultured at 37°C under 5% CO_2. Curcumin was obtained from Sigma Aldrich (USA). The MTT assay was used to determine the cytotoxic effect of curcumin on human PC cells. Panc-1 and BxPC-3 cells were plated in 96-well plates at 5,000 cells per well in a volume of 100 µl for 24 h before treatment. Following incubation, the media were replaced with fresh ones containing various concentrations of curcumin (10-125 µM) for 24 h, and the cells were incubated at 37°C. Twenty-four-hour later, the MTT solution was added to each well and incubated at 37°C for 4 h. The resulting formazan crystals were dissolved with DMSO by pipetting, and the absorbance was read at 570 nm in an ELISA reader. Data of cell viability was calculated by GraphPad Prism 8.

Gene expression analysis

According to the MTT results, the less cytotoxic concentration of curcumin was determined as 25-35 and 45 µM in both cell lines. Also, in this study, the treatment groups were compared with the control group. The control group was Panc-1 and BxPC-3 without any treatment with curcumin. Total RNAs were extracted from the curcumin-treated and control cells using TRIdity G reagent (Applichem, Germany). Reverse transcription was performed using a cDNA synthesis kit (2-steps RT-PCR kit, RTPL12, Vivantis, Malaysia). The effect of curcumin on *MTATP6, LDH, GLUT- 1, CYP2E1, CYP3A4,* and *β-Actin* (a reference gene for normalization) genes expression levels was determined using a real-time PCR technique with proper primers for each gene. Primer sequences of all genes were shown in Table 1. RT-qPCR was performed using SYBR green master mix (Thermo Fischer Scientific, USA) applying real-time PCR (Roche, Light Cycler 96 Real-Time PCR Cycler). The $2^{-\Delta\Delta CT}$ method was used to analyze the foldchanges in gene expressions.

Table 1. Primer sequences

Gene Name	Direction	Primer sequence (5'-3')
MTATP6	Forward	CCCCTCTATTGATCCCCACC
MTATP6	Reverse	AATGAGTGAGGCAGGAGTCC
LDH	Forward	TTGGTCCAGCGTAACGTGAAC
LDH	Reverse	CCAGGATGTGTAGCCTTTGAG
GLUT-1	Forward	CCGCAACGAGGAGAACCG
GLUT-1	Reverse	GTGACCTTCTTCTCCCGCATC
CYP2E1	Forward	GCAACCCGAGACACCATTTT
CYP2E1	Reverse	GCACACACTCGTTTTCCTGT
CYP3A4	Forward	GAGCTGAGATTGCACCACTG
CYP3A4	Reverse	TCGAGACAGTTGGGTGTTGA
B-Actin	Forward	ACTCTTCCAGCCTTCCTTC
B-Actin	Reverse	ATCTCCTTCTGCATCCTGTC

Biochemical analysis

Measurement of MTATP6, LDH, CPR, and MnSOD levels

$1x10^6$ cells/T75 plate were seeded and incubated for 24 hours. The Panc-1 and BxPC-3 cells were treated with three different concentrations (25-35-45 µM) of curcumin for 24 h, and later the cells were washed with PBS, trypsinized, and centrifuged to obtain pellets, which in turn were lysed with RIPA buffer (Santa Cruz, sc-24948) supplemented with a protease inhibitor cocktail (Roche, Switzerland). The BCA protein assay kit (Thermo Fisher Scientific,USA) was used for the analysis of the total protein concentrations of the groups. The supernatant portions of the samples were separated and collected into Eppendorf tubes. Biochemical analyses were performed according to the manufacturer's procedure. Analyses of MTATP6 (MyBioSource, cat no: MBS9325043), LDH activity (Elabscience, cat no: E-BC-K046), CPR (Elabscience, cat no: E-EL-

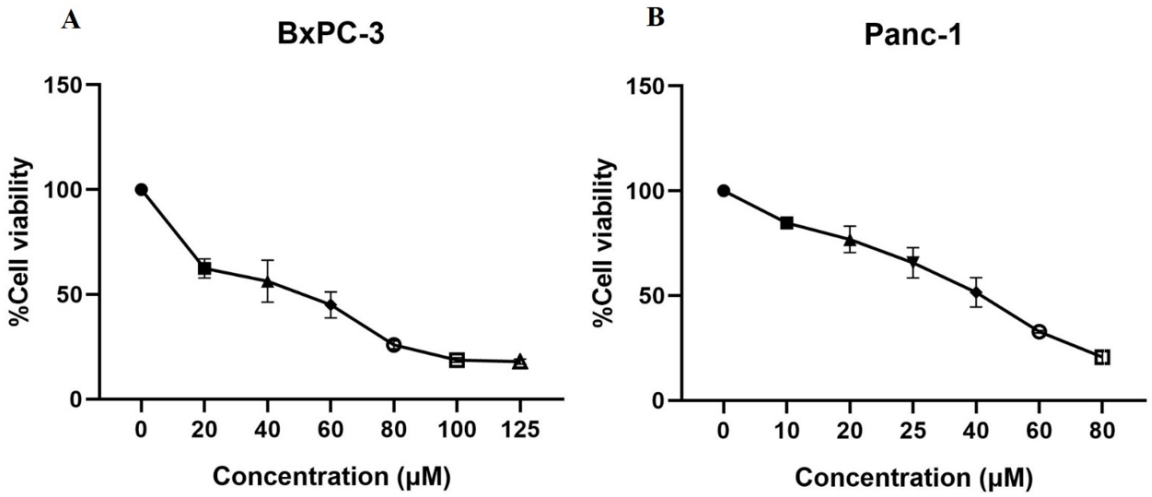

Figure 1. Dose-dependent effect of curcumin on the growth of BxPC-3 and Panc-1 cells for 24 h. Relative cell growth was measured after treatment with indicated curcumin concentrations for 24 h. A. BxPC-3, B. Panc-1 cells.

H0359), and MnSOD (Abcam, cat no: ab178012) were determined by ELISA methods. Protein analyses were performed using a colorimetric commercial test kit (Thermo, cat. no: 23227) at 562 nm wavelength and calculated as mg/mL. The results of mtATP6 were calculated as ng/mL, LDH activity was determined as U/mg protein, and CPR, MnSOD concentrations were calculated as ng/mg protein. The detection range of the mtATP6 is 0.625 - 20 ng/mL. Intra-assay and inter-assay CV is<15 %. The detection range of the LDH is 6-1,000 U/L. Intra-assay CV is1.8 % and the inter-assay CV is <2.4. The detection range of CPR is 0.16 -10 ng/mL. Intra-assay and inter-assay CV is <7 %. The detection range of the MnSOD is0.22 - 50 ng/mL. Intra-assay precision CV is <3.8 % and the inter-assay precision CV is<4.2%. All analyses were conducted with ELISA Reader BMG LABTECH (Germany) and Rayto Microplate washer (RT-2600, China).

Measurement of ATP, ADP, AMP levels and calculation of energy charge

Curcumin-treated cells were homogenized with RIPA buffer, centrifuged for 15 min at 10,000 ×g at 4°C, and then filtrated through a 0.2-mm syringe filter. The supernatant was stored at -80°C until the analyses were performed. ATP, ADP, and AMP were measured by the HPLC (HPLC-Shimadzu; LC-20AD) UV/VIS detector at a wavelength of 254 nm. The analytical column was 4.6 x 250 mm (Inertsil ODS-3V, 5µm). The mobile phase was 160 mM KH_2PO_4 with 100 mM KCl at pH 6.5. ATP, ADP, and AMP peaks were identified according to the corresponding retention times and confirmed by 'spiking' with added exogenous ATP, ADP, and AMP standards, respectively (ATP cat no: Sigma A7699, ADP Cat no: Ambresco 0160-20G, and AMP cat no: Sigma A1752). Concentrations of ATP, ADP, and AMP were calculated from the standard curve. The cellular energy charge was calculated as ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]) (36).

GEPIA2 database analysis

GEPIA2 (Gene Expression Profiling Interactive Analysis 2) database (http://gepia2.cancer-pku.cn/) is a quotable resource for gene expression analysis based on tumor and normal samples from the TCGA and GTEx databases (37). It was used to compare the gene expression levels of *mtATP6, LDHA, SLC2A1 (GLUT-1), CYP2E1,* and *CYP3A4* in pancreatic ductal adeno (PAAD) carcinoma compared to normal cells.

UALCAN database analysis

UALCAN (http://ualcan.path.uab.edu/index.html) is an effective web resource that can be analyzed based on relevant cancer data using the TCGA database. In this study, UALCAN was used to observe the association of target gene expression with patient survival (38).

Statistical analysis

The results were obtained from at least three independent experiments. The data comparison between curcumin-treated and control groups was performed using GraphPad Prism 8 (La Jolla, CA).

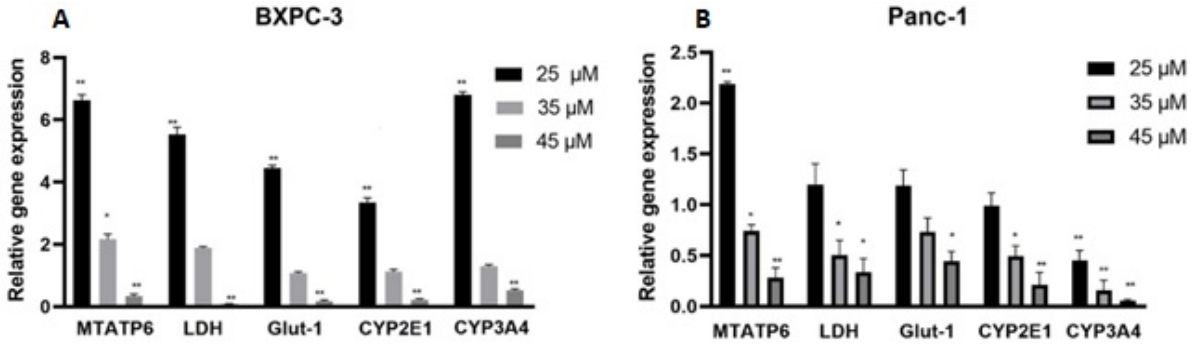

Figure 2. The relative gene expression level of *MTATP6, LDH, GLUT-1, CYP2E1* and *CYP3A4* in A. BxPC-3 and B. Panc-1 cells. To determine these gene expression levels, BxPC-3 and Panc-1 cells were treated with 25 µM, 35 µM and 45 µM Curcumin for 24 h.Gene expression was normalized to β-Actin mRNA levels. Different doses of curcumin supplementation groups were compared with the control group. The results shown are representative of the three independent experiments (* $P \leq 0.05$ and ** $P \leq 0.001$, treatments groups vs the control group).

One-way ANOVA with post-hoc Tukey HSD) test was used for multiple comparisons. In all analyses, p≤0.05 and p≤0.001 were considered statistically significant. For GEPIA2 database analysis, the p-value was generated using Student's t-test, and it was considered significant at p≤0.05. $|Log_2FC| > 1$ indicates the expression of a gene between cancer and normal cells. For UALCAN database analysis, the p-value was generated using Student's t-test and was considered significant at p≤0.05.

RESULTS

Effects of curcumin on cell viability

In this study, it was noticed that curcumin had a cytotoxic effect on BxPC-3 and Panc-1 cell lines through decreasing their proliferation when the curcumin concentration increased.

The effect of the 24-hour treatment with different curcumin working doses on the cell viability of the BxPC-3 cell line is shown in Figure 1A. Accordingly, at the end of 24 hours, the cell viability was respectively 62.43%, 56.32%, 47.79%, 44.57%, 25.95%, 18.74%, and 17.98% upon using 20 µM, 40 µM, 50 µM, 60 µM, 80 µM, 100 µM, and 125 µM of curcumin (p ≤0.05).

The cell viability percentages in the Panc-1 cells after 24 h administration of curcumin were 84.64% upon using 10 µM, 76.87% for 20 µM, 65.69% for 25 µM, 51.56 % for 40µM, 32.80% for 80 µM, and 20.74% for 60µM (Figure 1B).

IC_{50} values for 24 hours were found to be 47.26 µM in BxPC-3 cells and 45.84 µM in Panc-1 cells. Based on the IC_{50} values, 25 µM, 35 µM, and 45 µM curcumin doses were chosen as treatment doses in both cell lines.

Effect of curcumin on target genes expressions

After curcumin treatments, *MTATP6, LDH, GLUT- 1, CYP2E1, CYP3A4*, and β-Actin expressions were determined using RT-qPCR analysis. After 24-h of curcumin treatment to the BxPC-3 cell line, the gene expressions were compared to those of the control group. The results showed (Figure 2) that the *MTATP6* expression in the BxPC-3 cells was significantly increased when the cells were treated with curcumin at 25 µM (p≤0.001) and 35 µM (p≤0.05), whereas there was a significant decrease at 45 µM (p≤0.001) compared to the control group. In the Panc-1 cell line, the *MTATP6* expression level was significantly increased at 25 µM (p≤0.001) after curcumin treatment, but there was a significant decrease at 35 µM (p≤0.05) and 45 µM (p≤0.001) compared to the control group. When the doses were compared with each other, the treated groups showed significant results with each other in both BxPC-3 and Panc-1 cells (p≤0.001).

Additionally, the *LDH* expression level (Figure 2) was significantly increased at 25 µM curcumin concentrations, whereas it was decreased at 45 µM in BxPC-3 compared to the control group and within all treatment groups (p≤0.001). Concerning the *LDH* gene expression in Panc-1 cells, its levels decreased significantly (p≤0.05) at 35 µM and 45 µM curcumin treatment compared to the control group. There was an important difference between 25 µM-35 µM curcumin application groups and 25 µM-45 µM curcumin doses in both cell lines (p≤0.05, p≤0.001).

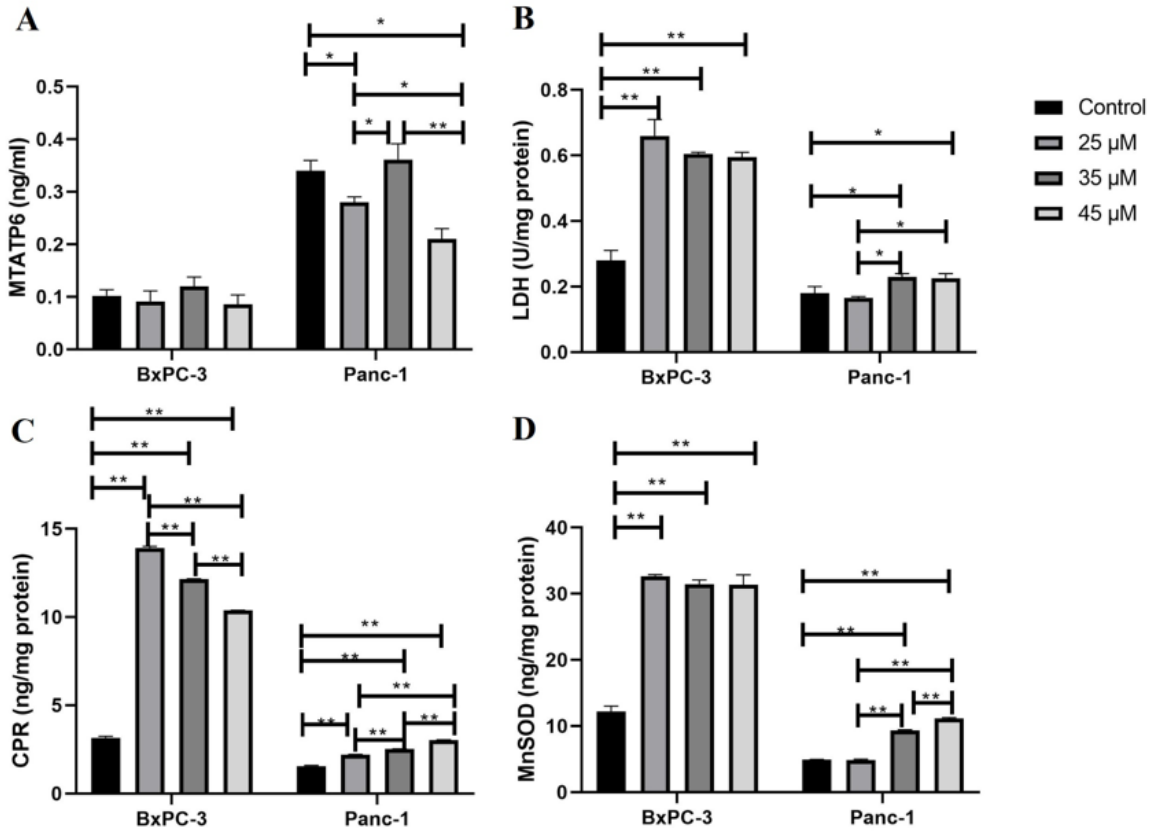

Figure 3. The effect of 25,35 and 45 µM curcumin treatment on the level of some proteins in pancreatic cancer cells A. MTATP6 B. LDH activity C. CPR levels D. MnSOD levels (*P≤0.05 and ** P≤0.001, treatments groups vs control group).

The gene expression level of *GLUT-1* was significantly increased at 25 µM curcumin concentration, whereas it was decreased at 45 µM in BxPC-3 cells compared to the control group and multiple comparisons (p≤0.001) (Figure 2). Similarly, the *GLUT-1* expression level was significantly decreased at 45 µM curcumin (p≤0.05) compared to the control group in Panc-1 cells. Also, there was a significant difference between 25 µM and 45 µM curcumin doses (p≤ 0.001).

For the *CYP2E1* gene, at 25 µM curcumin treatment, it significantly increased as compared to the control group (p≤0.001) (Figure 2). The decrease detected at 45 µM curcumin doses was statistically significant compared to the control group in BxPC-3 cells (p≤0.001). When comparing the dose groups of different concentrations with each other, the differences were significant in both BxPC-3 and Panc-1 cells (p≤0.001 and p≤0.05, respectively). In the Panc-1 cell line, a decrease in *CYP2E1* gene was observed in 35 and 45 µM dose groups (p≤0.05 and p≤0.001, respectively) compared to control group

The *CYP3A4* gene was upregulated at 25 µM (p≤0.001) and downregulated at 45 µM (p≤0.001) in the BxPC-3 cell line compared to the control. There was a significant difference when all of the treated groups were compared with each other (p≤0.001). All treated groups showed downregulation of the *CYP3A4* gene in the Panc-1 cell compared to the control group (p≤0.001). When the treated groups were compared with each other, there was a significant difference between 25 µM-35 µM and 25 µM-45 µM (p≤ 0.05) in the Panc-1 cell line.

The effects of various doses of curcumin-treatment on MTATP6, LDH, CPR, and MnSOD levels

As is seen in Figure 3A, the MTATP6 values of the control group and groups treated with 25 µM-35 µM-45 µM of curcumin did not show significant differences in BxPC-3 cells. In Panc-1 cells; a decrease was found between the control and the group treated with 25 µM and between the control and 45 µM group (p≤0.05). MTATP6 values were statistically significant between 25-35 µM treated

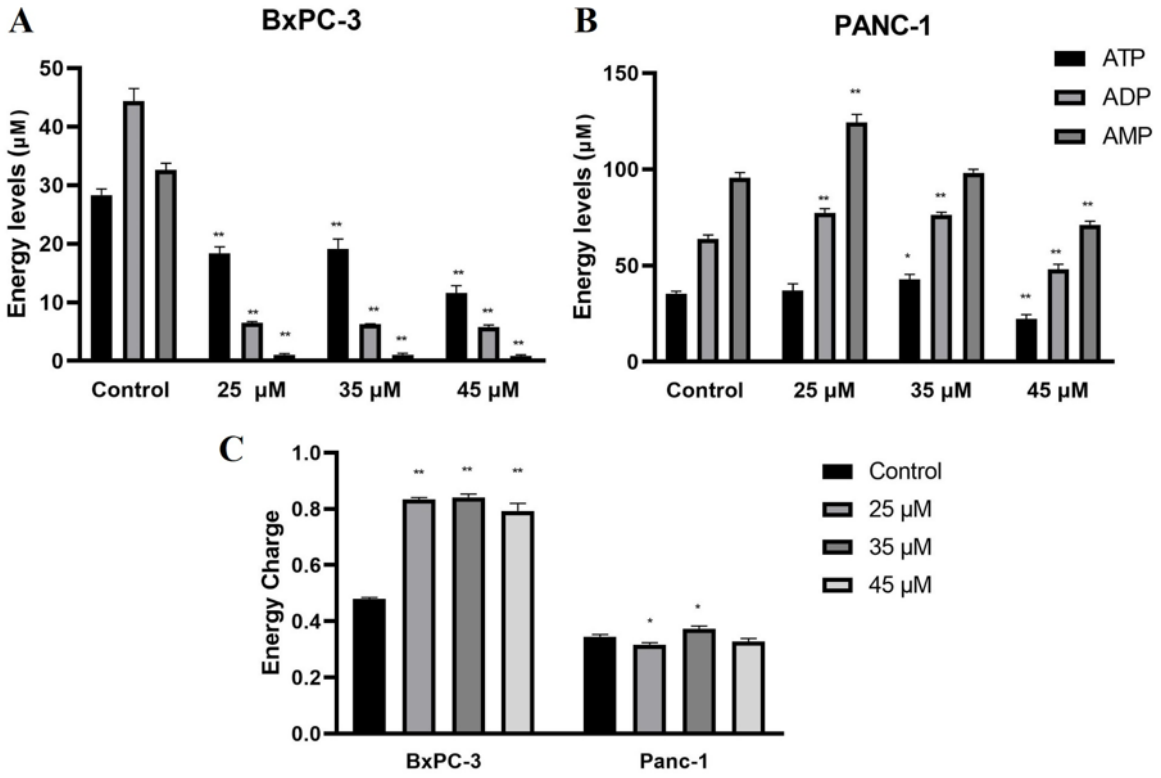

Figure 4. Comparison of the effects of 25,35 and 45 µM curcumin doses on the energy levels in A. BxPC-3 and B. Panc-1 cells C. Energy charge in both cells (*P≤0.05 and ** P≤0.001 compared to the control).

groups and between 25-45 µM treated groups (p≤0.05), and between 35-45 µM groups (p≤0.001).

LDH activity in BxPC-3 cells was higher in all treated groups compared to the control group (p≤0.001). In Panc-1 cells, LDH activity increased at 35 µM and 45 µM treated group compared to the control group (p≤0.05). There was an important difference between 25-35 µM groups, between 25-45 µM groups (p≤0.05), and also 35-45 µM groups (p≤0.001) (Figure 3B).

Significant difference was found in CPR values of both cell lines between the control and treated groups and among the treated groups (p≤0.001). In Panc-1 cells CPR levels increased in parallel with the increase in the dose of curcumin comparing to control group and each dose groups, whereas in BxPC-3 cells CPR levels decreased in parallel with the increases in the dose of curcumin comparing to 25 - 35 and 35-45 and 25-45 µM groups(Figure 3C). In addition, in BxPC-3 cells CPR levels increased in all treated groups compared to control group.

When comparing the control with all of the treated groups (p≤0.001) regarding the MnSOD values; significant increases were found in BxPC-3 cells. In Panc-1 cells; the highest level occurred in the group treated with 45 µM curcumin compared to other doses of curcumin and the control group. There was a significant difference when the treated groups were compared with each other (p≤0.001) (Figure 3D).

The effects of various doses of curcumin-treatment on energy regulation

The ATP levels in the BxPC-3 cell line decreased significantly in various treated groups compared to the control (p≤0.001). There was also statistical significance (p≤0.001) between the 25-45 µM and 35-45 µM treated groups (Figure 4A). The ATP levels in the Panc-1 cell line increased at 35 µM compared to the control group (p≤0.05), while a decrease was observed at 45 µM. There was also statistical significance (p≤0.001) between 25-45 µM and 35-45 µM treated groups (Figure 4B).

When the ADP levels were evaluated in comparison to the control and all treated groups in the BxPC-3 cell line, a significant decrease was observed (p≤0.001) (Figure 4A). The ADP levels in the Panc-1 cell line increased at 25 and 35 µM curcumin doses (p≤0.001) and decreased at 45 µM (p≤0.001) compared to the control group. There was also statistical significance

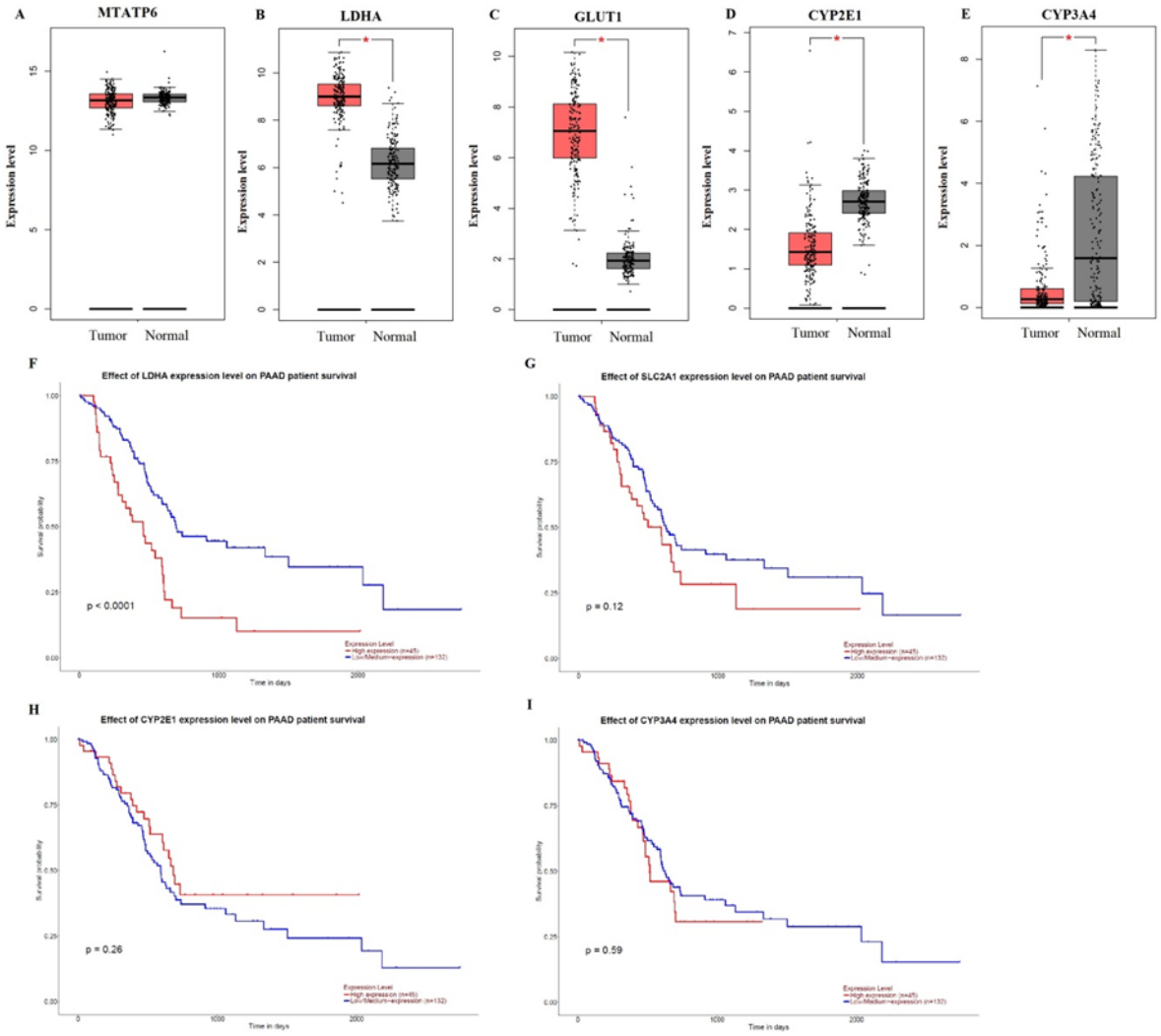

Figure 5. Analysis of target genes in pancreatic cancer differential expression analysis. A. *MT-ATP6, B. LDHA, C. SLC2A1 (GLUT-1),* D. *CYP2E1 and E. CYP3A4* genes in pancreatic cancer (179 tumor cases and 171 normal cases). GEPIA2 generates a box plot showing gene expressions in pancreatic cancer and normal tissues (TCGA tumor versus TCGA normal + GTEx normal). UALCAN plot shows the patient-survival relationship with the gene expressions of F. *LDHA* G. *SLC2A1 (GLUT-1)*, H. *CYP2E1* and I. *CYP3A4*. Blue line: low-expression groups (132 cases), red line: high-expression groups (45 cases).

(p≤0.001) between the 25-45 µM and 35-45 µM treated groups (Figure 4B).

The AMP levels in all of the treated groups in BxPC-3 cells were significantly decreased compared to the control group (p≤0.001). In the Panc-1 cell, there was an increase in the 25 µM curcumin-treated group and a decrease in the 45 µM treated group (p≤0.001) compared to the control. Significant difference was found between all of the treated groups (p≤0.001).

While the energy charge values increased in BxPC-3 cells at all doses compared to the control (p≤0.001), there was also a statistical significance (p≤0.05) between the 25-45 µM and 35-45 µM treated groups. In the Panc-1 cell, the energy charge decreased at 25 µM and increased at 35 µM (p≤0.05) compared to the control. There was also a statistical significance (p≤0.001) between the 25- 35 µM and 35-45 µM treated groups (Figure 4C).

GEPIA 2 and UALCAN databases

According to data analysis in GEPIA 2 databases, a high level of gene expression at the protein level of *LDHA* (*p*≤0.05, Figure 5B) and *GLUT1* (*p*≤0.05, Figure 5C) were observed in tumor compared to the normal tissues, while no significant difference was observed in the gene expression change of *MTATP6* (Figure 5A) protein. *LDHA* and *GLUT1* were upregulated in pancreatic tumor compared to the

normal tissues. However, a low level of gene expression at the protein levels of *CYP2E1* ($p \leq 0.05$, Figure 5D) and *CYP3A4* ($p \leq 0.05$, Figure 5E) were observed tumor compared to the normal tissues. Comparable data were obtained with ELISA and qRT-PCR.

Regarding data analysis in the UALCAN database, *LDHA, GLUT1, CYP2E1,* and *CYP3A4* gene expressions in pancreatic cancer were increased in tumor tissue at different stages compared to the normal tissue. However, there were no data on *MTATP6* gene expression from pancreatic cancer patients in the database. Bioinformatics analysis showed no significant association ($p>0.05$, Figures 5G, 5H, 5I) between *SCL2A1, CYP2E1, and CYP3A4* gene expression and pancreatic cancer patient survival, whereas up-regulated *LDHA* ($p \leq 0.0001$, Figure 5F) gene expression predicted a poor prognosis in pancreatic cancer patient survival.

DISCUSSION

Curcumin, 1,7- bis (4-hydroxy-3methoxyphenol)- 1,6-heptadiene3,5-dione, is a natural compound extracted from roots of *Curcuma longa* and exerts anti-proliferation, anti-invasion and anti-metastasis effects in various human cancers (39). Today, treatment with curcumin becomes a novel therapeutic alternative in cancer treatment. Several signaling pathways have been proven in curcumin treatment, but the mechanisms underlying the anti-cancer effects are still not fully explained. Since there is no study completely similar to our study, we could not directly compare our findings. Therefore, we compare our results with the studies using different agents or different cell lines.

Evaluation of energy status

MTATP6, as a nuclear regulatory element, regulates mitochondrial F_1F_0-ATP synthase activity to produce more ATP (40). In a study investigating the effects of Hesperidin and Chlorogenic acid alone and in combination on the MCF-7 breast cancer cell line, Hesperidin (100 µm) and Chlorogenic acid (350 µm) applied alone caused 20% and 50% cell death, respectively. When applied in combination, cell death was found to be 68.22%. As known, Hesperidin and chlorogenic acid have important effects on oxidative phosphorylation, mitochondrial dysfunction, and the sirtuin signaling pathway. In the presence of Hesperidin and chlorogenic acid, the synthesis of both ATP and lipid was downregulated. To highlight the effects of the estrogen receptor pathway on the transcription, translation, and synthesis of ATP in mitochondria, the researchers have shown that *CYC, TFAM, mtATP6, ATP5PB,* and *mtDNA* gene expressions were significantly reduced by combined therapy. In addition, there was no significant increase in ROS, but a decrease in ATP levels was observed in the treatment of MCF-7 cells with hesperidin and chlorogenic acid alone or combination. (41). As is seen in Figure 3A, MTATP6 ELISA results did not show any statistical importance in BxPC-3 cells, whereas in Panc-1 cell lines the MTATP6 levels demonstrated significant difference in all of the treated groups. When the results of both gene expression and the concentrations of *MTATP6* were evaluated, the findings of this study pointed to the occurrence of post-translational modifications in *MTATP6.* This finding is highly important given that an expression of mitochondrial *ATPase6* gene varies in different cancer types of the same tissue. Thus, it is important to clarify the changes in either the function or the localization of the protein in additional studies.

Both inadequate energy supply and increased oxidative stress may resultas a pathological phenotype.Furthermore, metabolic adaptations are sufficient to support proliferation under glucose-rich conditions when the amount of ATP-synthase falls below 30%(42). These findings also suggest to our results. According to our result we can conclude that curcumin treatment can contribute to the protection and provision of energy levels by increasing expression of *MTATP6* and energy charge. Curcumin showed positive effects on the requirement of the energy in the BxPC-3 cells.

Dijk et al. (2020) have investigated the role of mitochondria at Doxycycline/Gemcitabine combination therapy in the A549 cell line. As a result, a decrease in mitochondrial-encoded proteins, respiration, and membrane potential, and an increase of reactive oxygen species were found in A549 cells treated with doxycycline. Cellular ATP levels did not change. In contrast to the decreased synthesis of most mtDNA-encoded polypeptides, the synthesis of the ATP synthase subunits MTATP6 and MTATP8 was markedly increased in doxycycline-treated cells (43). Our results are similar to the findings of Dijk et al except for our Panc-1 cell's findings. The energy states of the Panc-1cell line differ from BxPC-3 cells. Therefore, we think that this difference may be related to the malignancy of the cell lines. In the evaluation of

energy charge, BxPC-3 cell's energy charge was higher in all of the treated groups than in the control group. We concluded that the energy charge did not depend on doses whether using a high or low dose of curcumin in BcxPC-3 cells. The Panc-1 cell line ATP was higher in both 25 and 35μM curcumin-treated groups. Briefly, our inferences are similar with Matsui et al. (1994).The researchers concluded that the energy charge provides the cells with a sensitive intracellular control mechanism and has an important role in the regulation of many enzymatic reactions whichutilizate ATP (44).

Evaluation of *LDH* and *GLUT-1* expression and LDH enzyme activity

Ma et al. (2020) studied cultured Panc-1 cells in 25 mM and 5 mM glucose media and treated with or without metformin. They observed that metformin significantly inhibited proliferation and viability and induced apoptosis in the Panc-1 cells, this effect was clearer in the low-glucose than in the high-glucose group. Metformin up-regulated the expression of miR-210-5p in low glucose, but not in high glucose. miR-210-5p, in turn, reduced the activity of PFK1 and LDH. The above researchers suggested that the high glucose group cells could make a compensatory adaptation to the stress-induced by metformin through increasing glucose consumption. However, due to the limited glucose supply and the high dependence on anaerobic glycolysis of cells in the low glucose group, they couldn't make effective adaptive compensation. Therefore, cells in the low-glucose group were more vulnerable to the toxicity of metformin. In conclusion, the enhanced inhibitory effect of metformin on the Panc-1cells cultured in low glucose may be due to the up-regulation of the expression of miR-210-5p, then inhibiting anaerobic glycolytic flux and inducing energy stress via repressing the expression of p-PFKFB2 and activity of LDH (45). In our study, the *LDH* expression level (Figure 2) was significantly increased at 25 μM curcumin concentrations, whereas it was decreased at 45 μM in BxPC-3 compared to the control group and within all treatment groups ($p \leq 0.001$). We noticed that *LDH* gene expression levels decreased at 35 μM and 45 μM curcumin treatment in the Panc-1 cells ($p \leq 0.05$) compared to the control group. There was an important difference between 25 μM and 35 μM curcumin application groups and between 25 μM and 45 μM curcumin doses in both cell lines. The findings that *LDH* gene expression decreased at a concentration of 45 μM in both cell lines confirm the relationship of high doses of curcumin with glucose consumption.

Glucose transporter *(GLUT)*-1 is highly expressed in malignant tumors and is considered a mediator to induce cancer metastasis (46). Liu et al (2019) show that aspirin (2 mM and 4 mM / 24h, 48h and 72h) modulates glucose uptake by downregulation of *GLUT-1*, this leads to inhibition of hepatoma cell proliferation). In addition, aspirin significantly reduces ROS levels and glucose consumption in hepatoma cells (). Interestingly, they found *GLUT-1* and HIF-1α were reduced by aspirin in HEPG2 and H7402 cells They determined that PDTC (pyrrolidine dithiocarbamate), which is an NF-κB inhibitor, affects glucose consumption levels in HEPG2 and H7402 cells via suppressing *GLUT-1* expression. They indicated high levels of *GLUT-1* correlate with poor recurrence-free survival of HCC (Hepatocellular carcinoma) patients.Furthermore, overexpression of *GLUT-1* in HepG2 cells was found to inhibit PDTC-induced or aspirin-induced inhibition of glucose metabolism (46).

In hypoxic conditions, after treatment of CD18 and S2-013 human pancreatic cancer cells with apigenin (0-50 μM, 24h), a decrease in *HIF-1α, GLUT-1 and VEGF* protein expression and mRNA levels was observed by Melstrom et al (2011) They postulated that Apigenin blocked the hypoxia-mediated induction of these three proteins in both cells (47). In a study conducted by Liao et al. (2015) transfected a constricted pcDNA3.1-GLUT1 vector into A549 cells. In the *in vivo* part of the study, tumor weight and metastatic rate were evaluated in nude mice bearing un-transfected, vector-transfected and pcDNA3.1-GLUT1 transfected A549 cells originated tumors (39). They found (39) that curcumin showed significant cytotoxicity against the proliferation effect at 45 μmol/L, and the *GLUT-1* expression was up-regulated in pcDNA3.1-GLUT1 transfected A549 cells Curcumin has been suggested to inhibit invasion of lung cancer by suppressing GLUT1/MT1-MMP/MMP2 signaling (39). Abouzeid et al. (2013) suggested that the treatment of HCT-116 cells with anti-Glut1 antibody-curcumin and anti-Glut1 antibody-curcumin+Doxorubicin micelles provided survival with tumor inhibition effect (48). A significant decrease in the glucose transporter *SLC2A1* (*GLUT-1*) mRNA expression was observed in both cell lines after BME (bitter melan extract) treatment in human oral cancer cell lines (Cal27 and JHU022) (49).

Additionally, after BME treatment, it was observed that PFKP (PFK1-phosphofructokinase 1 isoform), PKM (Pyruvate kinase isoform), LDHA (lactate dehydrogenase-A), and PDK3 (Pyruvate dehydrogenase kinase) protein expression were significantly reduced (49). In our study, at dose of 45 µM curcumin there was a decrease in *GLUT-1* gene expression in both cell lines. Although cancer types and the substances applied are different from the above mentioned studies (39, 48, 49), our findings showed similar results in curcumin treatment as with other agents. Therefore, we conclude that the effects of curcumin on *GLUT-1* are significantly important at a dose of 45 µM concentration.

Evaluation of MnSOD enzyme activity

It was shown that Genistein significantly suppressed cell viability in H460 and A549 lung cancer cells (50). MnSOD and FoxM1 overexpression antagonized the effects of genistein (40 µM), whereas MnSOD and FoxM1 knockdown enhanced the inhibitory effects of genistein (20 µM) in cancer stem-like cells (CSLCs), which characterize non-small cell lung cancer cells (LCSLCs). Therefore, the data showed that genistein suppressed CSLC properties via the modulation of MnSOD expression (50). In another study, curcumin has been shown to increase MnSOD activity in MIN6 pancreatic beta cells and Het-1A cells (51, 52). Schiffman et al. (2012) demonstrated that the cells received MnTBAP or curcumin oil pretreatment showed increased MnSOD expression compared with control untreated cells (52). The other important findings suggested that curcumin which has anti-lipotoxic effects protected MIN6 pancreatic β-Cells against apoptosis through activation of Akt, inhibition of nuclear translocation of FoxO1, and mitochondrial survival pathway (51). In a study, it was observed that Isovitexin (ISOV) inhibited carcinogenicity in hepatic carcinoma stem-like cells (HCSLC) via inhibition of MnSOD by FoxM1 (53). Treatment with isovitexin reduced the protein levels of MnSOD and FoxM1 (53). ISOV/thiostrepton combined treatment reduced protein expression of MnSOD, CD133, and FoxM1 and suppressed tumor growth in HCSLC xenograft models (53). In another study similar to this, it was shown that ISOV downregulates MnSOD and FoxM1 and suppresses their roles in invasion and migration (54). The suppressive effects of ISOV on the invasion and migration capabilities and epithelial-mesenchymal transition (EMT) phenotype can be potentiated by MnSOD or FoxM1 knockdown in HCSLCs and attenuated by MnSOD or FoxM1 overexpression in HCC cells (54). Wei et al. (2017) indicate to the promising anticancer effect of ascorbic acid that is dependent on cell properties, such as the basal redox state of the cancer and normal cells. The same study also revealed cell-dependent ROS generation in ascorbic acid treatment and identified the RelB-SIRT3-MnSOD axis as a critical contributor to ascorbic acid-induced radio-sensitization of cancer cells and radioprotection of normal cells. Pretreatment with ascorbic acid significantly decreased intracellular ATP when combined with predominant prostate cancer therapy, ionizing radiation (IR), while increased both extracellular and intracellular lactate production in the PC3 cells. Briefly, AA exacerbates mitochondrial dysfunction in cancer cells, while attenuating radiation-induced mitochondrial dysfunction in normal cells (55). In our findings, similar to other study findings, MnSOD activity increased with the addition of curcumin in both cell lines. Therefore, we conclude that, curcumin has an antioxidant property and an important role in maintaining cellular redox homeostasis.

Evaluation of CPR enzyme activity and CYP genes expression

Studies on the *CYP* genes expression demonstrated that IL-6 treatment significantly induced *CYP1B1* and *CYP2E1* gene expression, but did not affect *CYP1A1* gene expression in colorectal cancerHCT116 and SW480 cells (56). In a study on patients with breast cancer, high *CYP2E1* expression was shown to be associated with invasive lobular type tumor (57).

Patients with CYP3A4-negative *tumors* (63.2%) were found to have a higher rate of response to Docetaxel (DOC) than those with CYP3A4-positive tumors (26.1%). The researchers suggested that immunohistochemical measurement of CYP3A4 expression in the primary breast cancer lesion is useful for predicting the treatment response of tumors to DOC (58).The cytotoxic effect of *Clinacanthus nutans* plant methanol extract was investigated in many cancer cell lines, and the highest cytotoxic effect was observed in the HepG2 cell line (59). This plant extract has been reported to show significant inhibition of CYP3A4 and CYP2E1 activity in human liver microsomes ($p \leq 0.05$) (59). In our study, the decrease in *CYP2E1* expression detected at 45 µM curcumin doses was statistically significant as compared to the control group in BxPC-3 and Panc-1cells. All of the treated groups showed

downregulation of the *CYP3A4* gene in the Panc-1cell and at 45 μM in the BxPC-3 cells compared to the control group. Therefore, our findings are compatible with the other studies (58-59) Furthermore, the *CYP3A4* expression is generally higher in human breast cancer tissues compared to normal human breast tissue (25). The enzymatic activity of CYP3A4 was inhibited by using CO (carbon monoxide) and CO enhances sensitivity of breast cancer cells to PTX (Paclitaxel). Therefore the use of therapeutic CO in combination with PTX therapy was found to be a promising strategy to reduce or even prevent PTX resistance in human breast cancer (25).

NADPH-cytochrome P450 reductase (CPR) plays an essential role in the cytochrome P450 enzyme system, which detoxicates the endogenous and exogenous compounds in the metabolism (reference?). Therefore, the evidence that CPR enzyme levels increased with the treatment with curcumin in both cell lines can be said to be promising in treatment planning, as curcumin has a detoxifying effect according to our results.

CONCLUSION

Finally, the following conclusions can be drawn from the above data, including suppressing antioxidant status dramatically increases oxidative stress in the Panc-1and BxPC-3 cells. Treatment with curcumin inhibits oxidative stress by increasing MnSOD enzyme levels. This instance differed in both cell lines. While this change increased in a dose-dependent manner in Panc-1, the differences between the doses in BxPC-3 were similar. That is, BxPC-3 cells do not show dose-dependent manner in terms of antioxidant status.

One of our interesting findings is that mitocondrial ATP levels did not change in the BxPC-3 cells, but it showed an increase in the Panc-1 cells supplemented with curcumin. Furthermore, the dose-dependent increase in Panc-1 was observed in 35 μM concentrations mostly. The difference in the LDH levels between the groups of BxPC-3 cells treated with various doses of curcumin is insignificant, and changes of LDH level in Panc-1 cells indicate that it is dose-dependent. It can be said that the appropriate dose of curcumin is 45 μM for Panc-1 cell. The gene expression findings in Figure 5 confirm our findings of LDH expression in cancer. LDH gene expression decreases with the treatment at certain doses; however, values of enzyme did not support the gene expression findings. Therefore, we can explain this result by the occurrence of post-translational modifications. However, extensive studies are needed to fully elucidate that finding. The effects of curcumin on GLUT-1 are significantly important at a dose of 45 μM of curcumin, which affects glucose consumption in both cells.

Although the CPR enzyme level in the BxPC-3 cell line decreased in parallel with the increase in the dose of curcumin, it was found to be higher than the untreated group. So that, the concentration of curcumin at which CPR showed the best results in BxPC-3 cells was 25 μM. This was different for Panc-1, in which increasing curcumin dose resulted in an increase in CPR amount.Thus, curcumin can be concluded to have a detoxifying effect.

Curcumin treatment preserved energy load and supply energy charge. But this state differed in both cell lines. In the BxPC-3 cells, curcumin treatment reduced AMP, ADP and ATP levels comparing to the untreated cancer cells. In contrast, the Panc-1 cells showed an increase in ATP, AMP and ADP levels in all doses of curcumin compared to the untreated cells. Likewise, this finding was also reflected in the mitochondrial ATP value, which showed no change in the BxPC-3 cells, and a significant increase at the dose of 35 μM curcumin. It is well known that the utilization of ATP is regulated by the energy charge. This study suggests that the effect on the energy state is still unclear and differ in each cell line. Therefore, the Panc-1 and BxPC-3 cells showed different results that can be attributed to the aggression of the cells. Furthermore, curcumin has shown anti-proliferative, antioxidant effects and may be target for anticancer drugs.

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