The Potential Role of Nrf2-PD-L1 Axis in Promoting of Oxaliplatin Resistance in Colon Cancer Cells

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Keywords: Nrf2, PD-L1, Colorectal cancer, Oxaliplatin, Resistance, CD80

DOI: https://doi.org/10.21203/rs.3.rs-58926/v1

Abstract

Background: Nuclear factor–erythroid 2–related factor 2 (Nrf2) has a key function in promoting chemoresistance in various cancers. PD-L1 is one of the downstream targets of Nrf2 signaling pathway, having some beneficial impacts on tumors growth by inhibition of the immune system. The aim of this study was to investigate the potential role of Nrf2- PD- L1 axis in the promotion of oxaliplatin resistance in colon cancer cells.

Result: Our data revealed that Nrf2 and PD-L1 mRNA expressions were markedly higher in tumor tissues compared to margin tissues. PD-L1 mRNA expression level was also increased in the resistant cells. However, Nrf2 expression was decreased and increased in SW480/Res and LS174T/Res cells, respectively. Nrf2 inhibition through siRNA in SW480/Res and LS174T/Res decreased the IC50 values of oxaliplatin. Inhibition of Nrf2 significantly increased oxaliplatin-induced apoptosis and reduced migration in SW480/Res cells when accompanied with oxaliplatin.

Conclusion: Our study suggests that effective inhibition of Nrf2/PD-L1 signaling pathways can be considered as a novel approach to improve oxaliplatin efficacy in colon cancer patients.

1. Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide, which is routinely diagnosed at a higher rate of distant metastases [1]. Oxaliplatin resistance is a common phenomenon in the failure of these patients' treatment following the surgery [2]. Nuclear factor–erythroid 2–related factor 2 (Nrf2) plays a key role in the protection of cells against oxidative stress and inflammation in numerous tissues [3]. Nrf2 overexpression has been reported in diverse types of tumors, associated with disease-free and shorter overall survival [4-6]. Furthermore, abnormal activation of Nrf2 signaling pathway has been demonstrated in CRC [7,8]. Kelch-like ECH–associated protein 1 (Keap1) is an adaptor protein, involving in ubiquitination and degradation of Nrf2, and directly regulating Nrf2 expression [9]. In addition, Keap1 and Nrf2 have recently been introduced as effective factors in which their balanced functions are important for the prevention of oncogenesis in colon cells [10].

Programmed death-ligand 1 (PD-L1) molecule is classified as a member of the CD28-B7 family; which commonly expressed on the surface of active T cells [11,12]. PD-1/PD-L1 signaling prevents the production of cytokines including TNF-α, IFN-γ, and IL-2. This protein is actually a checkpoint in the immune system, which works with two mechanisms of agonizing apoptosis (a programmed cell death) in T lymphocytes and also a reduction of the apoptosis in the T regulator lymphocytes [12,13]. It has been reported that losing of PD-L1 through reduction of the antigen-recognition threshold and promoting the cytotoxicity activity of CD8+ T cells involves in the inhibitions of tumor growth and metastasis, and mice resistance to viral infection [13].

Recently, having promising clinical outcomes in different types of cancers, various antibodies against PD-1 or PD-L1 have gained massive attention [14-16]. However, resistance to these antibody-based

treatments has been reported by several studies [17,18]. To get rid of it, combined therapies for advanced melanoma are tested. Up to now, several upstream regulators of PD-L1 have been announced[19-21].

Identifying and developing alternative therapies for inhibiting PD-1 / PDL1 is necessary to overcome drug resistance in cancer patients. A new strategy replaces the use of NRF2 inhibitors. Yin and colleagues conducted a study in 2018 that NRF2 is upstream of the PD-L1 regulator. NRF2 destruction or suppression significantly increased the activity of both CD4 + and CD8 + cells to inhibit the progression of melanoma; NRF2 inhibition in combination with anti-PD-1 therapy increased the anti-tumor efficacy[22]. Altogether, all of these studies clarified that NRF2-targeted suppression has a strong rationale to be examined in cancer[23,24].

The aim of the present study was to investigate the potential role of Nrf2, PD- L1 and CD80 in the promoting of oxaliplatin resistance in colon cancer cells. First, we examined their expression in the tumoral and margin tissue samples from CRC patients. Then, we investigated the potential role of Nrf2-PD-L1 axis in promoting of oxaliplatin resistance in colon cancer cells. Finally, our results revealed that the effective inhibition of the Nrf2-PD-L1 axis can be considered as a promising approach for the treatment of colon cancer patients.

2. Materials And Methods

2.1 Patients and Specimens

This study was accomplished under the institutional bioethical guidelines of the Ethical Committee at Tabriz University of medical sciences (IR.TBZMED.VCR.REC.1397.403). Tumor and matched marginal tissues (N=50) were gathered during surgery from patients who referred to Imam Reza Hospital at Tabriz University of Medical Sciences. For this purpose, we obtained written informed consent from all patients. The clinical and pathological characteristics of all participants are brought in Table 1. We immediately transferred all tissue samples into RNAase inhibitor solation (Qiagen) and stored them at -80 ºC for further experiments.

2.2. Gene expression analysis through quantitative real-time PCR

The qRT-PCR technique was applied to assess gene expressions. First, total RNA of tumor tissues and cells were extracted by using RiboEx reagent (GeneAll, Seoul, Korea) according to the manufacturer's protocol. Then total RNA was quantified and qualified by NanoDrop Spectrophotometer (Thermo, USA) and agarose gel electrophoresis, respectively. Next, cDNA was synthesized through a cDNA synthesis kit (Takara, Japan). Finally, mRNA expressions were examined by the Roche light cycler system (Roche, Germany) by applying SYBR Premix Ex Taq (Takara, Japan). The primer sequences have been shown in Table 2. Relative Nrf2, PD-L1 and CD80 mRNA expressions in tissue samples were analyzed through the $2^{-\Delta\Delta Ct}$ method, which endogenous β actin was considered as an internal control [25]. However, for the assessment of raw data from the cell samples, we applied the Pffafl method [26] and considered GAPDH gene as an internal control. All of the experiments were performed at least in triplicate.

2.3. Cell culture:

SW480 and LS174T colon cancer cells were purchased from the National Cell Line Bank of Iran (Pasteur Institute, Tehran). SW480 and LS174T resistant cells were developed as previously described [27-29]. SW480 and SW480 resistant cells were cultured in RPMI-1640 medium; LS174T and LS174T resistant cells cultured in DMEM high glucose medium which all were supplemented with FBS 10% (Fetal bovine serum source commercial catno. 10437028). The cells were incubated in a humidified atmosphere containing 5.2% CO2 at 37 °C.

2.4. siRNA transfection

First, SW480, SW480/Res and LS174T, and LS174T/Res cells were seeded with a density of 5×10^5 cells per well in a six-well plate and allowed to reach to 60% to 70% confluency. Then, culture media were removed and siRNA transfection (Santa Cruz, CA) was performed by applying transfection reagent (Santacruz Biotechnology) according to the manufacturer's instruction. Briefly, siRNA (80 pmol) and transfection reagent (8 pmol) were separately diluted through siRNA transfection medium (Santacruz Biotechnology) and incubated at room temperature for 10 minutes. Then, they were mixed and incubated at room temperature for 20 minutes or more. Finally, the mixture was transferred to the wells comprising cells and transfection medium; the cells incubated about 6h at 37°C in a CO2 incubator. In the following, the cells were cultured in the RPMI-1640 medium containing 20% FBS. After 48 hours, the cells were used for further experiments.

2.5. Assessment of cells' viability through MTT assay

First, we seeded the cells in the 96-well plates with a density of 10^4 cells/well in triplicate. Then, the cells were incubated for 24h or overnight. In what follows, the media was removed and the cells were incubated with different oxaliplatin concentrations in the presence and absence of Nrf2 inhibitor (Nrf2 siRNA) alone and in combination. After 24h, the media was removed and replaced with fresh media containing 3-(4, 5- Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide solution (MTT) (2 mg/ml) and further the cells incubated for 4 h at 37 °C. Then, the media was replaced with 200 µl DMSO and followed with gentle shaking 15 min. Finally, a microplate reader (Sunrise, Tecan, Australia) was used to measure the optical density (OD) of the wells in 570 nm [25]; IC50 of drugs on the cells were calculated using Graph pad prism v.7 through non-liner regression procedure.

2.6. Apoptosis assay:

We applied annexin V/PI assay to examine the impact of Nrf2 siRNA on the oxaliplatin-induced apoptosis in SW480/Res cells. First, SW480/Res cells were seeded in a six-well plate (2×10^5 cells per well). Then, the cells were transfected with Nrf2 siRNA and treated with oxaliplatin for 48h. Further, the cells were harvested through trypsin EDTA 0.05%, subsequently washed twice with PBS, and stained by applying ApoFlowEx® FITC Kit (EXBIO, Vestec, Czech Republic). Finally, the apoptotic cells were analyzed through

MACS Quant 10 flow cytometry (Miltenyi Biotech, Germany). FlowJo (7.6.1) software was used for acquisition and analysis of raw data [30,31].

2.7. Woundhealing assay

Wound healing assay was applied to investigate the impact of Nrf2 siRNA on the SW480/Res cells' migration capacity. The cells with a density of 50, 000 cells per well were seeded into 24-well plate and incubated until to be 90% confluent or more in the monolayer. Then, to create "wound" the layer was scratched by applying sterile yellow micropipette tip and the cells' debris washed with serum-free media. After Nrf2 siRNA transfection, the wound images were captured by using the light microscope at different times including 0, 6, 12, 24, and 48 hours. The migration capacity was calculated by evaluating the distance between the wound edges through Image J software. A wound-healing assay was performed in triplicate[32,33].

2.8. Assessment of Superoxide Dismutase Activity:

Superoxide dismutase (SODs) plays a major role in antioxidant defence regulations by catalyzing the partitioning of superoxide free radical ($O2^{-}$) into molecular oxygen and hydrogen peroxide ($H2O2$). Therefore, the amount of enzyme in the cellular and extracellular environment is crucial for the prevention of oxidative stress damages. The experiment was subdivided into four groups: Nrf2 siRNA, oxaliplatin, the combination of Nrf2 siRNA + oxaliplatin and control. For this assay, according to the kit protocol (TEB PAZHOUHAN RAZI, Iran) SW480/Res and LS174T/Res cells with a density of 2×10^6 cells/ml were harvested for each group. These cells were Lysed by repeated cycles of freezing and thawing in ice-cold PBS (pH 7.2-7.4). Then, the mixture was centrifuged at 14,000 x g at +4°C for 5 minutes. After that, the supernatant was gathered and transferred to the fresh tubes and placed on ice. Finally, the samples and reagents were added to each well and the absorbance was read at 440-460 nm using a microplate reader[34,35].

2.9. Statistical analysis

All of the data were obtained at least three independent experiments or more. Data analysis was performed using one-way ANOVA or unpaired t-test tests through Graph Pad prism (v 10.00) software. We considered $p < 0.05$ as statistically significant.

3. Results

3.1. Nrf2, PD-L1, and CD80 gene expressions statues in colon cancer tissues

We applied qRT-PCR to examine the Nrf2, PD-L1 and CD80 gene expression levels in CRC tissues. Our results showed that Nrf2 and PD-L1 expressions were markedly higher in tumor tissues compared to margin tissues ($p < 0.001$). However, the difference in CD80 expression was not statistically significant in the groups ($p < 0.001$) (Fig. 1). Further, the correlation between Nrf2, PD-L1 and CD80 expressions was

determined through Spearman's rank coefficient test. Our results revealed a positive correlation between Nrf2 and PD-L1 gene expressions in CRC samples ($r=0.8434$, $p < 0.001$).

3.2. Different Nrf2 and PD-L1 expression levels in sensitive and resistant colon cancer cells

mRNA expression of Nrf2 and PD-L1 in sensitive (SW480 and LS174T) and resistant colon cancer cells (SW480/Res and LS174T/Res) were evaluated through qRT-PCR technique. Our findings revealed an almost two-fold decrease in Nrf2 and a four-fold increase in PD-L1 expressions in SW480/Res compared to sensitive ones. LS174T/Res in comparison with LS174T cells had higher Nrf2 and PD-L1 expressions almost three-fold and seventeen-fold, respectively (Fig. 2).

3.3. The impact of Nrf2 inhibition on PD-L1 expression

We applied different Nrf2 inhibitors (brusatol and Luteolin) to evaluate the impact of Nrf2 inhibition on PD-L1 expression. SW480 and LS174T sensitive and resistant cells were treated with brusatol (40 nM) and Luteolin (20 μM) alone and in combination for 24 h. Nrf2 inhibition by brusatol, luteolin, and their combination in SW480 cells decreased Nrf2 mRNA level about five, four and five-folds, respectively, which were ten, seven and ten decreases for PD-L1 expression. However, Nrf2 expression was increased about three-folds after its activation through tBHQ, which was three folds increase for PD-L1 (Fig. 3A-B). In addition, our results from SW480/Res cells showed that Nrf2 mRNA levels were decreased about thirteen, five and twelve folds after its inhibition by brusatol, Luteolin, and their combination, respectively. However, Nrf2 expression was increased about five-fold after applying tBHQ. In SW480/Res cells, Nrf2 inhibition by brusatol, Luteolin, and their combination caused five, Four, and Five folds decrease in PD-L1 mRNA level; PD-L1 expression was increased about three-folds in the tBHQ treated group (Fig. 3C-D). Brusatol, Luteolin, and their combination in LS174T cells decreased Nrf2 mRNA level about six, eleven and fourteen folds, respectively, and also decreased PD-L1 expression about five, eight, and five, respectively. However, Nrf2 expression was increased about three-fold after its activation through tBHQ, which increased PD-L1 expression about four folds (Fig. 4A-B). Furthermore, in LS174T/Res cells, Nrf2 inhibition by brusatol, Luteolin, and their combination caused eleven, ten and eleven-fold decreases in Nrf2 mRNA level, respectively. However, tBHQ increased Nrf2 expression about three-fold in the cells. Nrf2 inhibition by brusatol, Luteolin, the combination of brusatol and luteolin caused nine, eight and ten folds decrease in PD-L1 mRNA level PD-L1 expression was increased about three-fold in the tBHQ treated group (Fig. 4C-D).

3.4. The impact of Nrf2 siRNA on Nrf2 suppression

We evaluated the impact of siRNA- mediated Nrf2 inhibition on Nrf2 and PD-L1 expressions in the sensitive (LS174T and SW480) cells using qRT-PCR. We calculated relative Nrf2 gene expression in comparison with the control group which was considered as 100%. The cells were transfected with different dosages (40, 60, and 80 pmol) at different times (24, 48, and 72 h). Our results showed that mRNA expression of Nrf2 was decreased 77.06%, 54.10%, and 78.95% at 24, 48 and 72 h after the transfection, respectively ($p < 0.05$) (Fig.5). In addition, Nrf2 siRNA transfection decreased Nrf2

expression almost 88.87%, 71.13%, and 47.25% at 40, 60, 80 pmol of Nrf2 siRNA concentrations, respectively, suggesting that Nrf2 siRNA acts in dose-dependent manner ($p < 0.05$). Fortunately, NC siRNA treatment had marginal impacts on Nrf2 expression versus to control group. We obtained 80 pmol of Nrf2 siRNA at 48 h as the best knocking down point for further experiments after examining the impact of Nrf2 siRNA on Nrf2 suppression at different times and doses.

3.5. Nrf2 silencing caused downregulation in Nrf2 and PD-L1

To find the impact of Nrf2 silencing on Nrf2 and PD-L1 expression, SW480, SW480/Res, LS174T and LS174T/Res colon cancer cells were transfected with 80 pmol of Nrf2 siRNA for 48h. Then, the gene expressions were evaluated through qRT-PCR. Our results showed that by Nrf2 silencing, Nrf2 and PD-L1 expressions were down-regulated about 51%, 55%, 36% and 49.5% in the SW480, SW480/Res, LS174T and LS174T/Res cells, respectively ($p < 0.05$) (Fig. 6 A). In addition, with this transfection, PD-L1 expression was decreased around 51%, 55%, 36%, and 49.5% in the SW480, SW480/Res, LS174T and LS174T/Res cells, respectively ($p < 0.05$) (Fig. 6B).

3.6. Nrf2 suppression caused a reduction in cell survivability:

To evaluate the impact of Nrf2 inhibition on oxaliplatin efficacy to overcome oxaliplatin resistant in colon cancer cells, we applied two oxaliplatin resistant colon cancer cells (SW480/Res and LS174T/Res cells). The cells were transformed with Nrf2 siRNA or NC siRNA and treated with different oxaliplatin concentrations for 48h. Then, the IC50 values of the oxaliplatin and Nrf2 siRNA alone and their combination were determined through MTT assay. Our results showed that the IC50 values of oxaliplatin in SW480 and LS174T cells increased from 10.56 and 8.83 μM to 89.60 and 43.34 in resistant cells, respectively. Nrf2 inhibition by siRNA in SW480/Res and LS174T/Res decreased the IC50 values of oxaliplatin from 89.60 and 43.34 μM to 23.08 and 12/25 μM, respectively ($p < 0.05$). Furthermore, IC50 values of Nrf2 siRNA were determined 34.22 and 10.29 μM for SW480/Res and LS174T/Res cells, respectively (Fig.7).

3.7. The impact of Nrf2 silencing on oxaliplatin-induced apoptosis

To investigate the impact of Nrf2 silencing on the oxaliplatin-induced apoptosis, we applied annexin V/PI analysis. SW480/Res cells were treated with oxaliplatin (90 μM) and Nrf2 siRNA (80pml) alone and in combination for 48h. The cells were stained with Annexin V/PI and apoptotic cells detected by flow cytometry. Our data showed that the apoptosis rates were 40.18 % (35.6 %+ 5.13%) for oxaliplatin, 4.47 % (2.74%+ 1.73%) for Nrf2 siRNA, and 40.22% (32.4 + 8.18) for combination of Nrf2 siRNA and oxaliplatin treated groups(Fig.8).

3.8. Nrf2 silencing decreased cell migration in SW480/Res cells:

We used a wound-healing assay to investigate the impact of Nrf2 silencing on migration in SW480/Res cells. First, the cells' monolayer was scratched and further treated with oxaliplatin (90 μM) and Nrf2 siRNA (80 pml) alone and in combination. Our findings showed that Nrf2 knocking down reduced

migration of the cells in the scratched area, which was more remarkable when accompanied with oxaliplatin (Fig.9).

3.9. Effects of Nrf2 siRNA on SOD activities in colon cell lines

In order to examine the impact of Nrf2 siRNA on SOD activities, we used the Superoxide Dismutase activity assay kit. The formula for Inhibition ratio of SOD (%) is:

Inhibition ratio of SOD (%) = (Ablank1 – Ablank3) – (Asample – Ablank2) X 100

(Ablank1 – Ablank3)

A = Absorbance

when we used the combination of Nrf2 siRNA + Oxaliplatin in SW480/Res and LS174T/Res colon cancer cells, the significant reduction was observed in the activity of SOD versus to the control, siRNA, and Oxaliplatin groups ($p<0.001$). Our results revealed that the inhibition ratio of SOD was 65%, 83%, and 52%, respectively for Nrf2 siRNA, oxaliplatin, and their combination in SW480/Res cells. Furthermore, the inhibition ratio of SOD in LS174T/Res cell for Nrf2 siRNA, oxaliplatin, and their combination was 71%, 87%, and 48%, respectively (Fig.10).

4. Discussion

Since the past 30 years, massive advances have been developed for the treatment of colon cancer patients, which alleviated the prevalence of the disease, but, its growing death rate still is statistically remarkable [36]. During the past decades, tremendous efforts have been done in cancer biology to clarify the complicated molecular mechanisms of malignant progression in various types of cancers, which have established effective progresses in molecular target therapy of cancer. Colon cancer patients have been suffering from chemotherapy-resistance especially oxaliplatin resistance. Therefore, finding effective molecular targets underlying chemoresistance to overcome this dilemma is an urgent need [37]. Recently, it has been reported that Nrf2 and PD-L1 signaling pathway had co-regulatory functions in melanoma cancer. It has also been shown that NRF2 regulated PD-L1 expression, which can be considered as a potential alternative strategy for PD-1/PD-L1 antibody-based treatment of melanoma [22]. Up to now, the function of the Nrf2-PD-L1 axis has not been clarified in the colon cancer especially in the oxaliplatin resistant cells.

Therefore, we aimed to clarify the importance of the Nrf2-PD-L1 axis in resistance and migration of colon cancer. First, we examined Nrf2, PD-L1, and CD80 genes expression in CRC biopsy specimens. Our results showed that Nrf2 significantly was overexpressed in CRC samples versus the margin tissues. Several studies which were conducted studies in colon cancer patients on colonoscopic tissue samples revealed that Nrf2 is overexpressed in the patients [38]. In addition, Nrf2 overexpression was concomitant with the stage and grade of tumors [39]. It has also been shown that Nrf2 overexpression has a vital function in the pathogenesis of CRC. Nrf2 up-regulation has also been shown in various tumors including gastric,

lung, breast, liver, and endometrium tumors tissues [40,41]. Nrf2 through induction of antioxidant transcription in pancreatic cancer promoted gemcitabine resistance [42]. The diagnostic value of Nrf2 overexpression has been reported for the prediction of esophageal squamous cell carcinoma [43]. Then, we investigated PD-L1 mRNA expression in CRC, which our results revealed that PD-L1was up-regulated in CRC tissues versus to margin tissues. It has been reported that several types of solid tumors by overexpressing of PD-L1 provide an immune shield in tumor microenvironment to overt T cell cytolysis such as colorectal, melanoma, RCC, ovarian, NSCLC, and thymoma tumors[44,45]. Furthermore, our data revealed no significant difference in mRNA expression of CD80 between tumoral and margin tissues, which was consistent with previous studies. We found a positive correlation between Nrf2 and PD-L1 mRNA expressions in tumor tissues. It has been shown that Nrf2 positively controlled PD-L1 expression [22].

Further, we examined Nrf2 and PD-L1 expressions in sensitive and resistant colon cancer cells. Our results showed that PD-L1 and Nrf2 expressions were increased in LS174T/Res cells versus to sensitive ones. In addition, the results revealed that LS174T/Res cells with higher PD-L1 and Nrf2 expression had higher oxaliplatin-resistance compared to SW480/Res cells. However, in the SW480/Res cells, PD-L1 and Nrf2 expressions were increased and decreased, respectively. It has been previously reported that Nrf2 stability might be increased in SW480/Res cells through Nrf2- Her2 or Nrf2-Her2/p62 axis interactions [46,47]. Nrf2 overexpression has been demonstrated in oxaliplatin resistant colon cancer cells including SW620-OX, and HCT116-OX[48]. Furthermore, it has been shown that doxorubicin-resistant colon spheres (HCT116) had higher Nrf2 expression [49].

Chemical inhibition of Nrf2 with Brusatol and Luteolin showed a strong reduction of Nrf2 and PD-L1 mRNA expression levels. This result supports the idea that Nrf2 positively controlled PD-L1 expression. Consistently, Nrf2 activation by tBHQ also strongly increased Nrf2 and PD. L1 mRNA expression levels. Sabzichi et al. showed that Nrf2 activation through 20 μM of tBHQ increased Nrf2 mRNA expressions [50]. Further, we studied the impact of Nrf2 inhibition on PD-L1 expression by applying Nrf2 siRNA. siRNAs are the powerful tools to silence target genes in mammalian cells which with specific knocking down of target genes are introduced as the amazing biological tools [51]. Our results revealed that Nrf2 silencing significantly decreased Nrf2 and PD-L1mRNA expressions in the sensitive and resistant cells in a time-dependent manner. In addition, we applied Nrf2 silencing to evaluate its potential impacts on oxaliplatin efficacy to break down oxaliplatin resistance in SW480/Res, and LS174T/Res cells. The results from MTT and apoptosis assays showed a strong synergistic effect of Nrf2 knocking down with oxaliplatin in both SW480/Res and LS174T/Res cells. Previously, we demonstrated that Nrf2 inhibition by Brusatol promoted oxaliplatin–induced cytotoxicity and apoptosis in the cells [29]. It has been reported that knocking down of NRF2-dependent antioxidant response by siRNA enhanced efficacies of different drugs including doxorubicin, etoposide, cisplatin, 5-FU, camptothecin, and gemcitabine in various cancers [52,48]. Duong et al. also reported that Nrf2 silencing through siRNA promoted the efficacy of 5-FU in different pancreatic cancer cells including PANC-1, AsPC-1, and COLO-357 [53]. It has been demonstrated that a recombinant NRF2-siRNA significantly downregulated the NRF2-dependent ATP-binding cassette (ABC) efflux transporters, and increased the sensitivity of MG63 and 143B cells to sorafenib, doxorubicin,

and cisplatin drugs [54]. Nrf2 suppression through siRNA increased ROS overwhelmed tumor growth and consequently promoted cell death through induction of sensitivity to chemotherapy [55]. Carboplatin in combination with Nrf2 suppression by naked siRNA duplexes inhibited tumor growth in lung cancer [55].

Nrf2 inhibition by luteilin in oxaliplatin resistant colon cancer cells strongly had a synergistic effect with different chemotherapy drugs including oxaliplatin, doxorubicin [48] and Nrf2 suppression through Brusatol sensitized resistant acute myeloid leukemia (AML) cells to arsenic trioxide (ATO), daunorubicin (Dnr), and cytarabine (Ara-c) drugs [56]. Knocking down or silencing of Nrf2 reduced phospho-AKT, induced P27, and consequently promoted doxorubicin cytotoxicity and induced apoptosis in SKOV3 ovarian cancer cell line [57,58].

Cancer cells through the migration process spread out to the distant tissues. Migration is considered an essential step for the spreading of malignancies which eventually leads to metastasis. There is an enormous mechanistic event underlying cellular invasion which makes tumor cells spread out to distance organs[59,60]. In the following, we examined the potential role of Nrf2 siRNA in inhibition of migration in SW480/Res colon cancer cells through wound-healing (scratch) assay. The results showed that treatment of SW480/Res cells with Nrf2 siRNA and Oxaliplatin alone or in combination significantly inhibited migration with maximum inhibition when the cells treated with a combination of Nrf2 siRNA and Oxaliplatin. Nrf2 inhibition by chrysin suppressed proliferation, invasion, and migration of glioblastoma cells [61]. Wang et al. reported that oridonin inhibited migration and invasion; it also promoted apoptosis in breast cancer cells. Furthermore, it has been shown that silencing of Nrf2 gene promoted the efficacy of chemotherapy and suppressed tumor growth in non-small cell lung cancer [62].

5. Conclusions

Our data revealed that Nrf2 positively regulated PD-L1 expression in SW480/Res and LS174T/Res colon cancer cells. Having an effective molecular function in promoting and maintaining of oxaliplatin resistance, Nrf2-axis can be suggested as an attractive candidate for adjuvant therapy in colon cancer. Altogether, the current study suggests that an effective inhibition of Nrf2 using specific siRNA can be a promising approach to overcome oxaliplatin resistance and migration in colon cancer patients.

Abbreviations

Nrf2: Nuclear factor–erythroid 2–related factor 2 (Nrf2), CRC: Colorectal cancer (CRC), Keap1: Kelch-like ECH–associated protein 1, PD-L1: Programmed death-ligand 1, tBHQ: Tert-buthylhydroquinone.

Declarations

Acknowledgments

The authors wish to thank Immunology Research Center, Tabriz- Iran and Shahid Sadoughi University of Medical Science, Yazd, Iran for their support by unrestricted free access to the web site for data collection.

Authors' contributions:

All authors participated in writing. Writing-review and language correction.

Funding: This work was financially supported by grants from the Iran National Science Foundation (INSF), Iran (project no. 97021150) and Tabriz University of Medical Sciences, Tabriz, Iran (project no. 61892). We gratefully acknowledge them for their contribution to this study.

Availability of data and materials:

Data presented in this manuscript is available upon request.

Ethics approval and consent to participate:

This article does not contain any studies with human participants or animals performed by any of the authors

Consent for publication:

All authors have read and approved the final version of the manuscript.

Conflict of interests

All authors declare no potential conflicts of interest.

Competing interests:

The authors declare that they have no competing interests.

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Figures

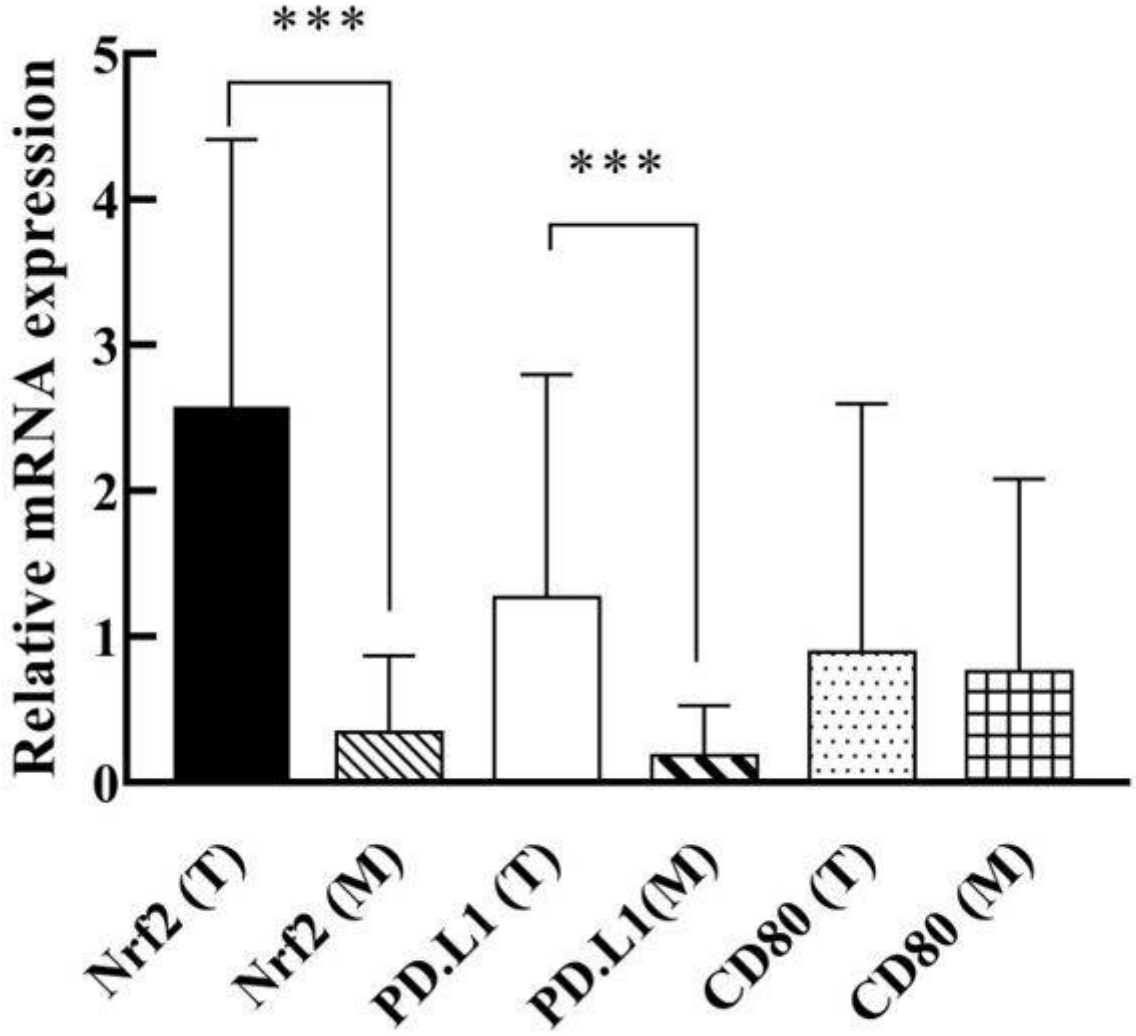


Figure 1

The mRNA expression level of Nrf2, PD-L1 and CD80 in tumor (T) and margin (M) tissues of colon cancer patients. Nrf2 and PD-L1 were significantly overexpressed in tumor tissues in comparison with margin ones ($p<0.001$). There was not any significant different in CD80 mRNA expression between tumor and margin tissues. The results were gathered at least from three independent experiments and finally expressed as mean ± SD. ***$p < 0.001$

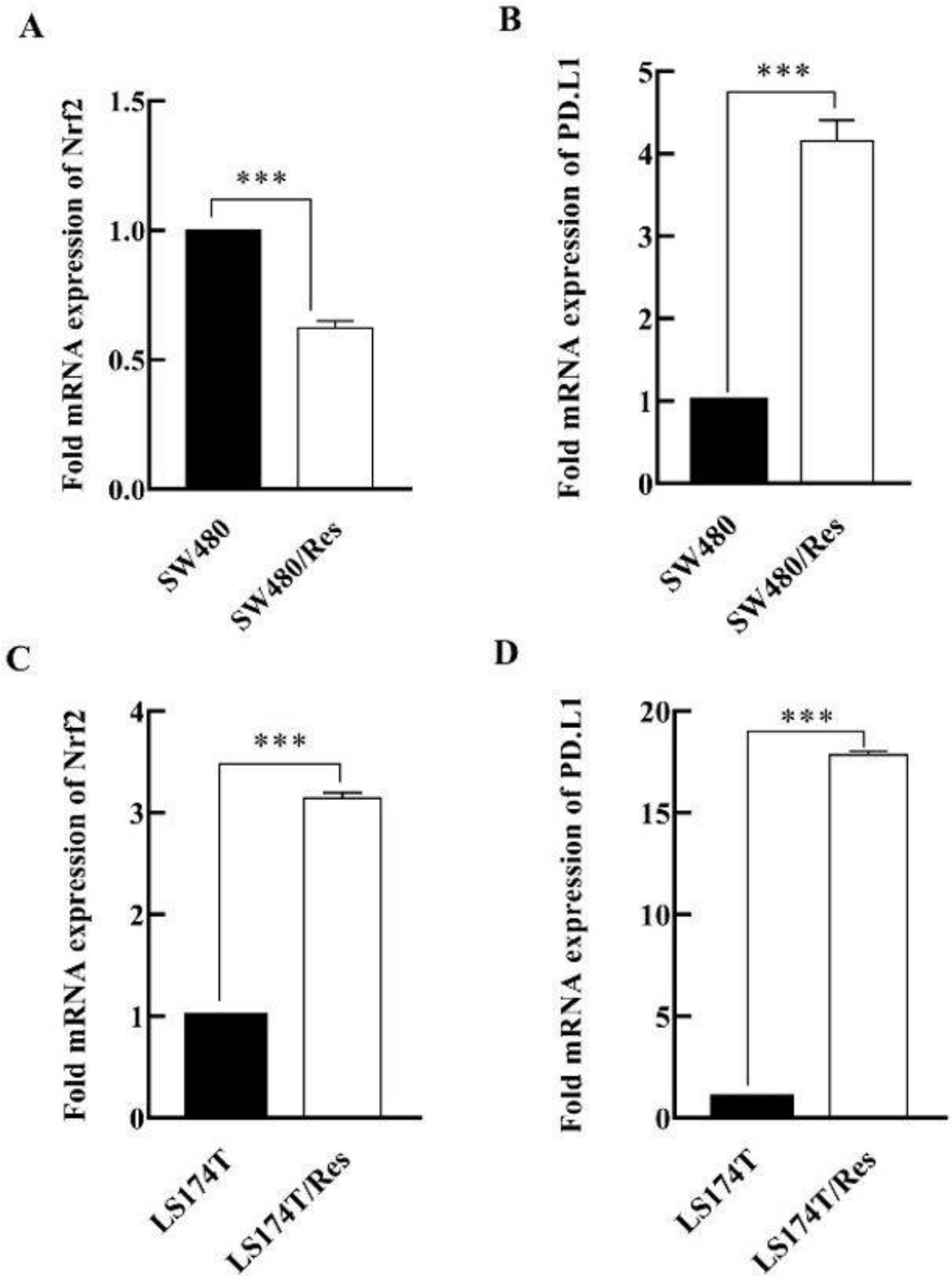


Figure 2

A–D, Nrf2 and PD-L1 mRNA expression levels in sensitive (SW4800 and LS174T) and resistant (Res) (SW480/Res and LS174T/Res) colon cancer cells. two-fold decrease in Nrf2 and four-fold increase in PD-L1 expressions in SW480/Res compared to sensitive ones (A and C). LS174T/Res in comparison with LS174T cells had higher Nrf2 and PD-L1 expressions almost three-fold and seventeen-fold (B and D). The

results were gathered at least from three independent experiments and finally expressed as mean ± SD. ***p < 0.001

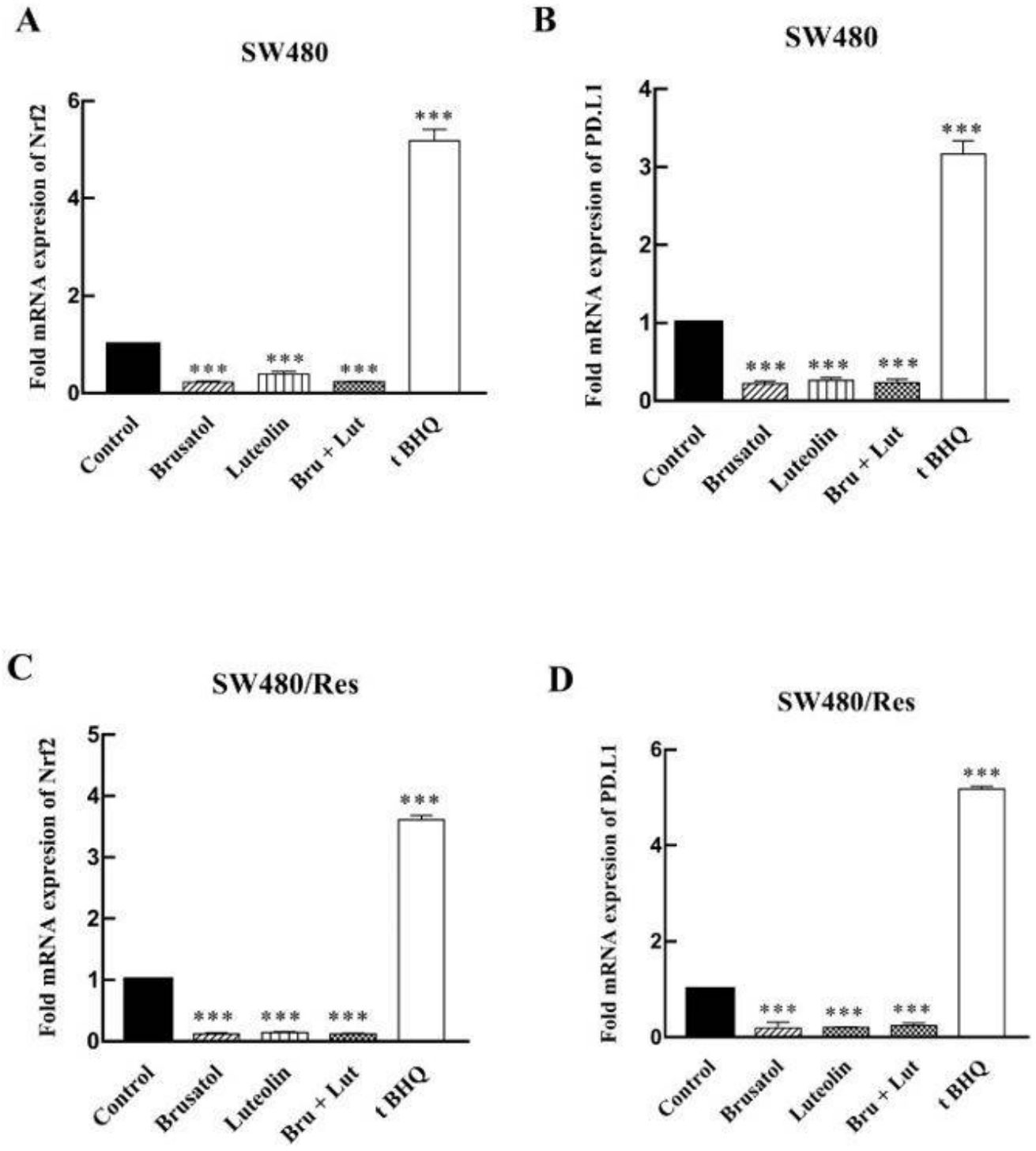


Figure 3

The impact of Nrf2 inhibition by Brusatol (Bru) and luteolin (Lut); and activation by Tert-buthylhydroquinone (tBHQ) on Nrf2 and PD-L1 mRNA expression levels. The sensitive and resistant (Res) SW480 colon cancer cells were treated with brusatol (40 nM) and Luteolin (20 μM) alone or in

combination; and Tert-buthylhydroquinone (20 μM) for 24 h. The results were gathered at least from three independent experiments and finally expressed as mean ± SD. ***p < 0.001.

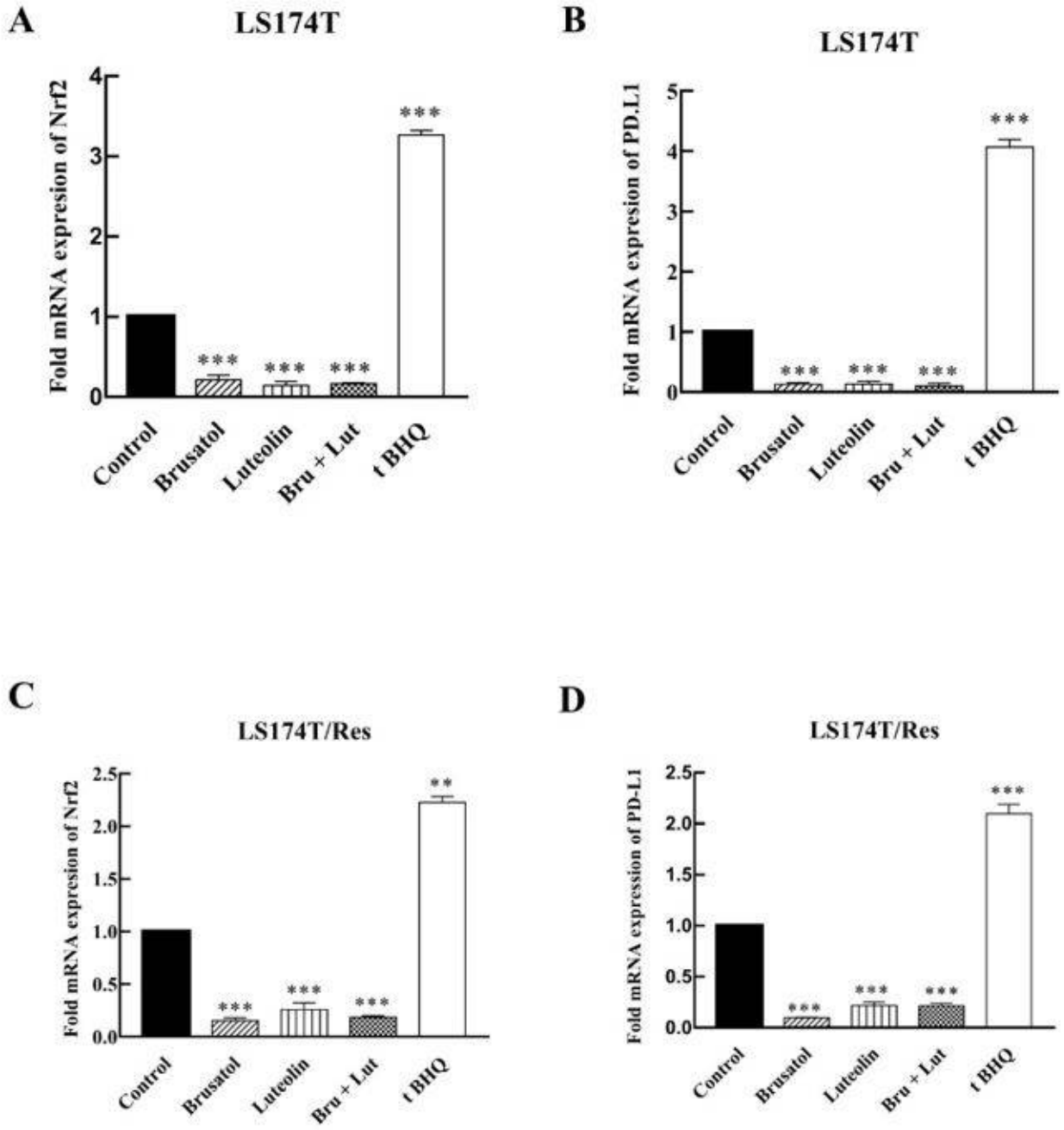


Figure 4

The impact of Nrf2 inhibition by Brusatol (Bru) and luteolin (Lut); and activation by Tert-buthylhydroquinone (tBHQ) on Nrf2 and PD-L1 mRNA expression levels. The sensitive and resistant (Res) LS174T colon cancer cells were treated with brusatol (40 nM) and Luteolin (20 μM) alone or in combination; and Tert-buthylhydroquinone (20 μM) for 24 h. The results were gathered at least from three independent experiments and finally expressed as mean ± SD. ***p < 0.001.

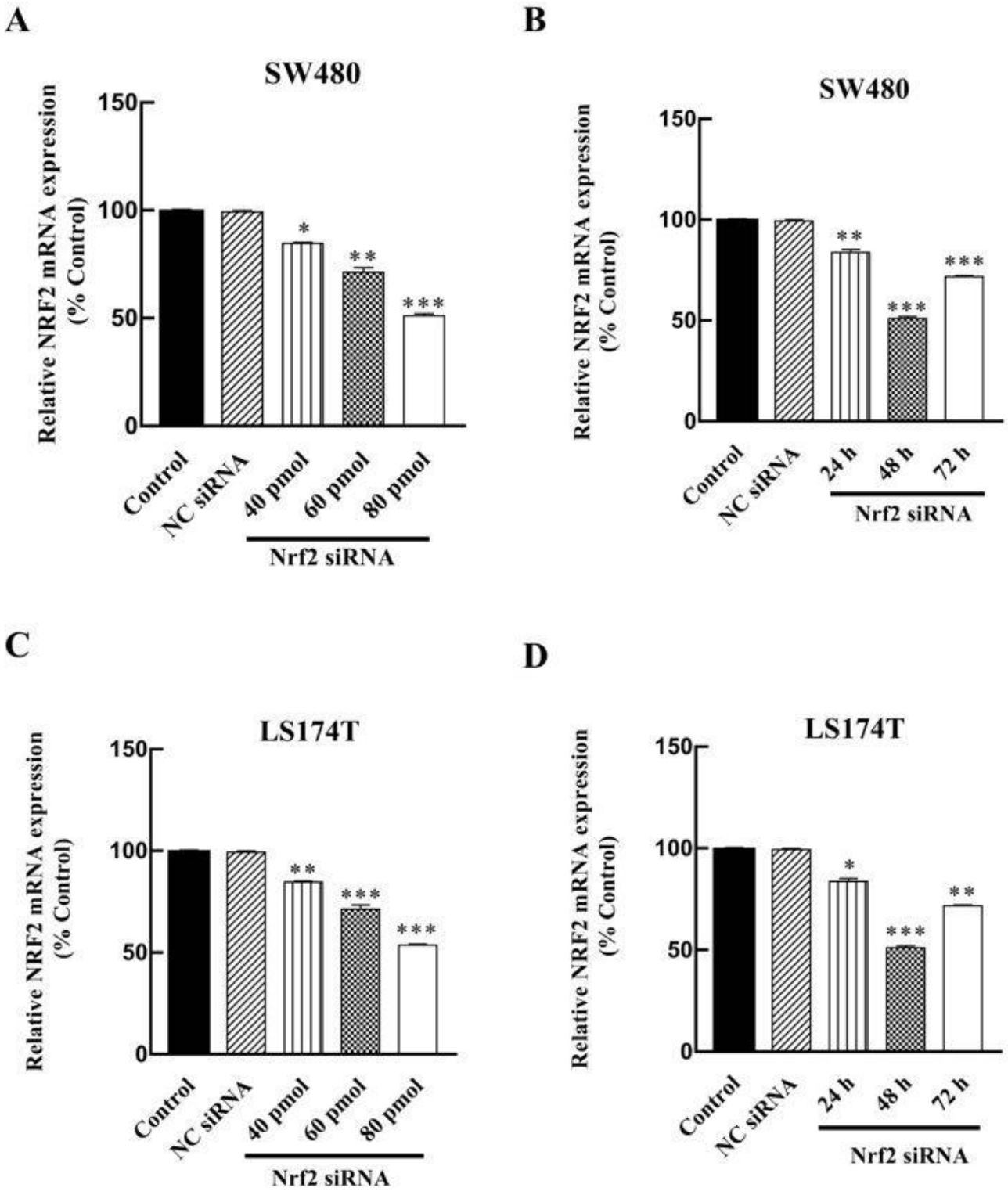


Figure 5

The impact of Nrf2 siRNA on Nrf2 expression in colon cancer cells. SW480 and LS174T cells were transfected with specific siRNA or (NC) siRNA in different concentrations including 40, 60, and 80 pmol for 24, 48, and 72 h; Nrf2 mRNA expression was examined through qRT-PCR. The results were gathered at least from three independent experiments and finally expressed as mean ± SD. ***p < 0.001.

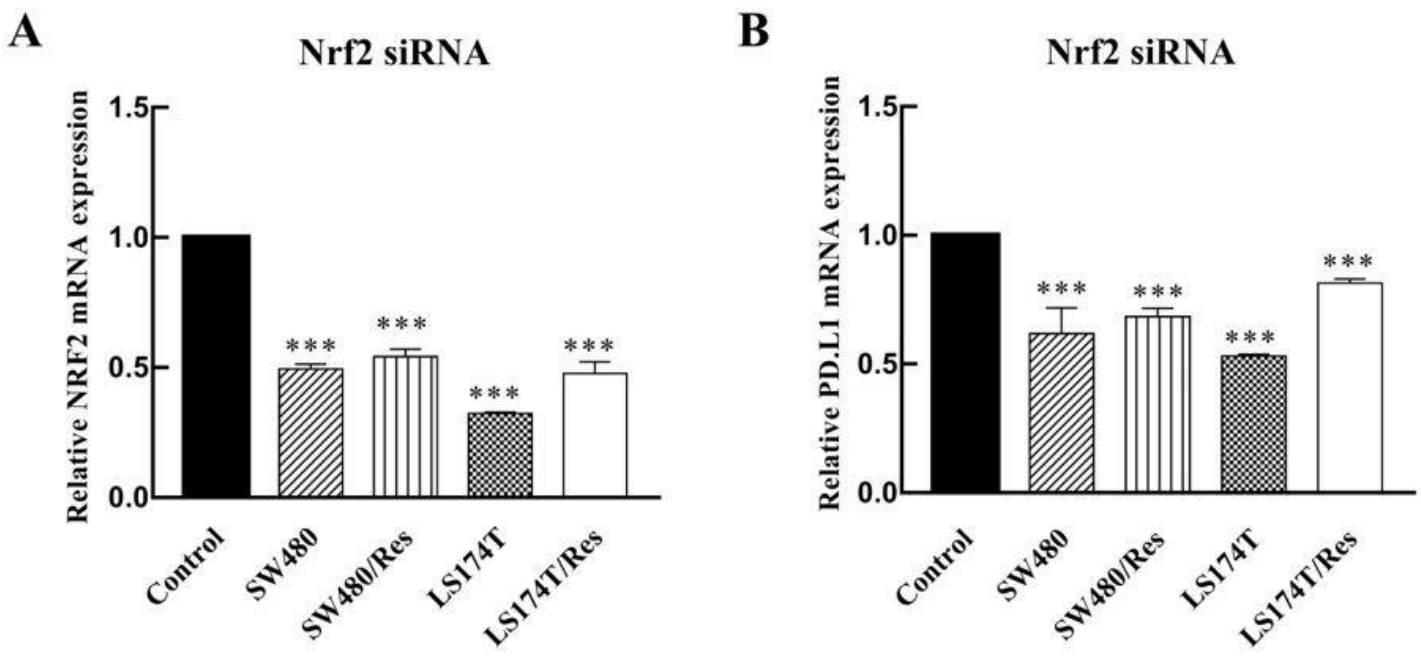
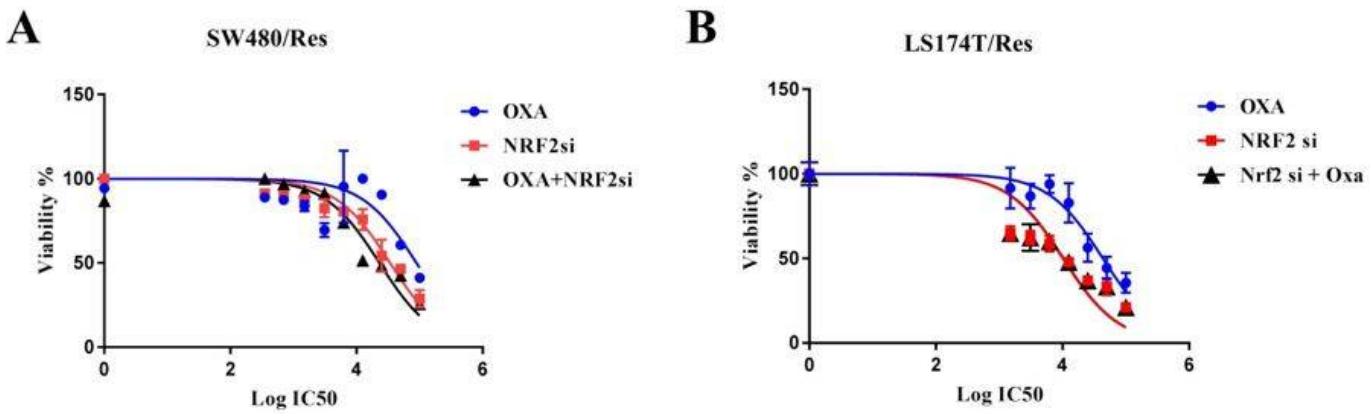


Figure 6

The impact of Nrf2 siRNA on Nrf2 and PD-L1 expressions. Sensitive (SW480 and LS174T) and resistant (SW480/Res and LS174T/Res) colon cancer cells were transfected with Nrf2 siRNA (80 pmol) for 48h. The results were gathered at least from three independent experiments and finally expressed as mean ± SD. ***p < 0.001.



	OXA	NRF2si	OXA+NRF2si
LogIC50	4.952	4.533	4.364
IC50	89442	34131	23100

	OXA	NRF2 si	Nrf2 si + Oxa
LogIC50	4.637	4.004	4.012
IC50	43337	10098	10287

Figure 7

The impact of Nrf2 siRNA on oxaliplatin efficacy in resistant colon cancer cells. SW480 and LS174T resistant colon cancer cells were transfected with Nrf2 siRNA (80 pmol) which was also followed by

different oxaliplatin concentrations for 48h. The impacts of Nrf2 siRNA on oxaliplatin induced cytotoxicity in SW480/Res (A) and of LS174T/Res (B) cells.

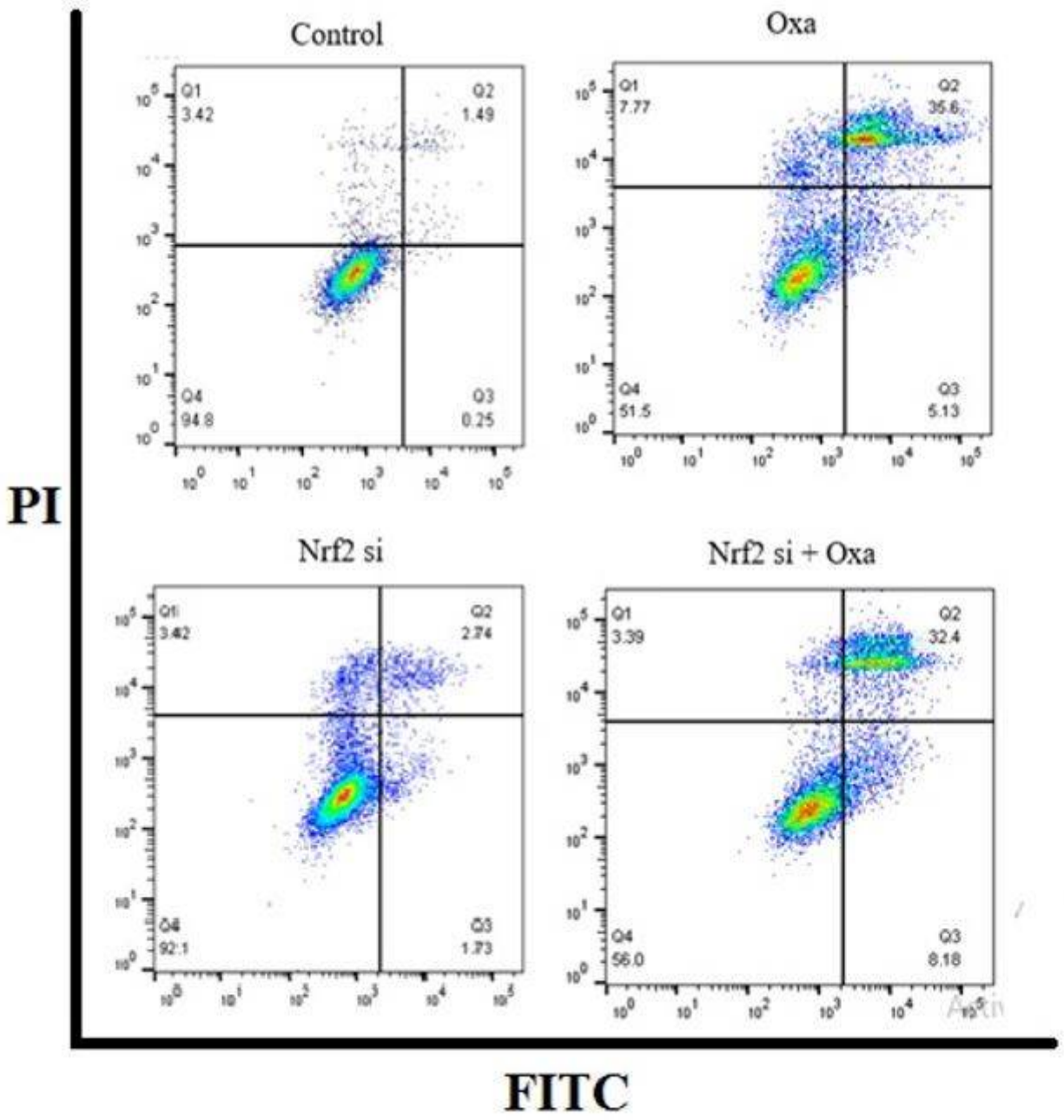


Figure 8

The impact of Nrf2 siRNA on oxaliplatin induced apoptosis in resistant in SW480 colon cancer cells. SW480 resistant colon cancer cells were transfected with Nrf2 siRNA (Nrf2 si) (80 pmol) which was also followed by oxaliplatin (Oxp) (89/60 μM) for 48h.

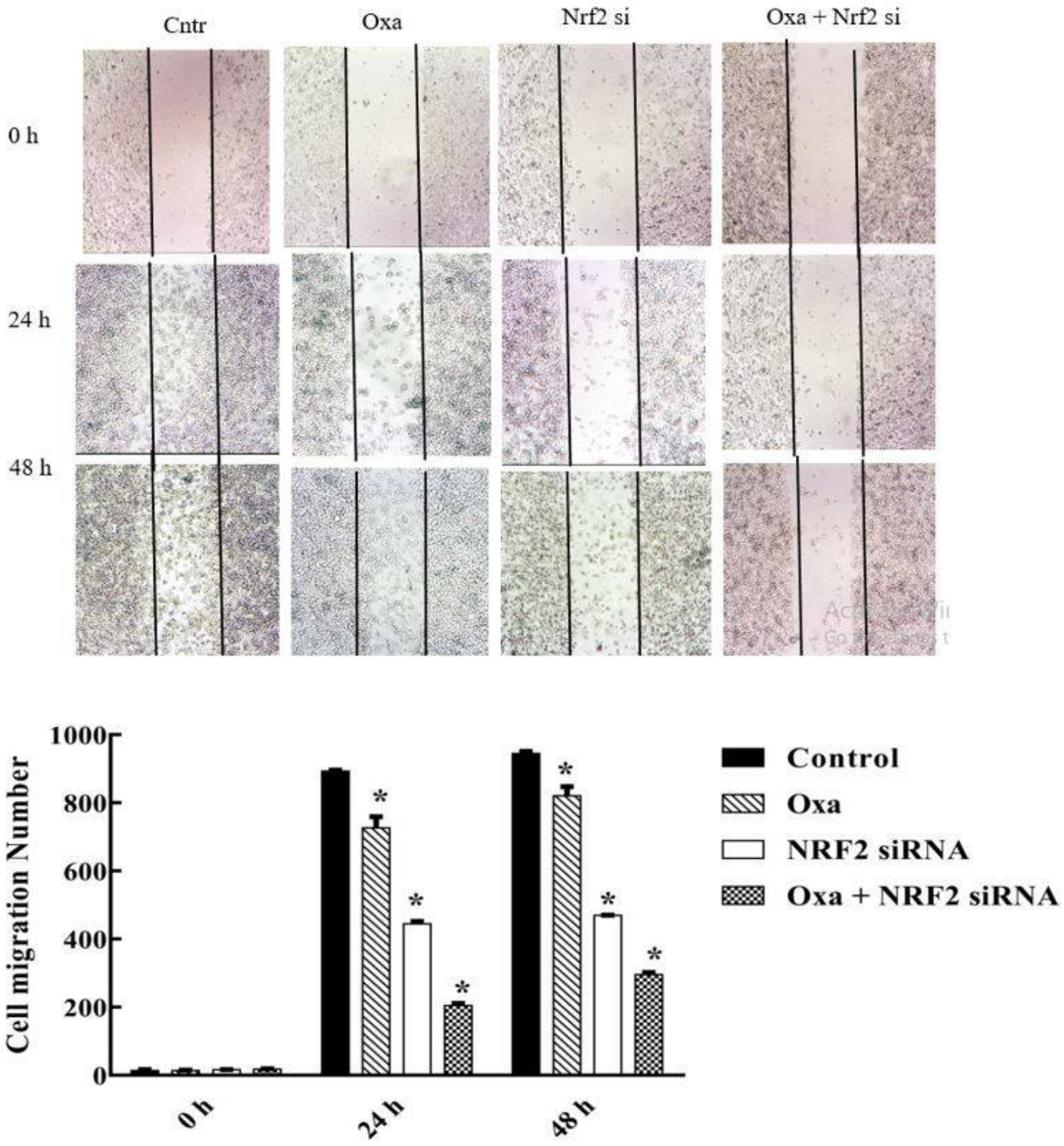


Figure 9

The impact of Nrf2 siRNA on reduction of migration in resistant SW480 colon cancer cells. SW480 resistant colon cancer cells were transfected with Nrf2 siRNA (Nrf2 si) (80 pmol) which was also followed by oxaliplatin (Oxp) (89/60 μM) for 48h. Then, the cells motility was monitored by wound healing assay for 0, 24, and 48 h. The results were gathered at least from three independent experiments and finally expressed as mean ± SD. *$p < 0.05$.

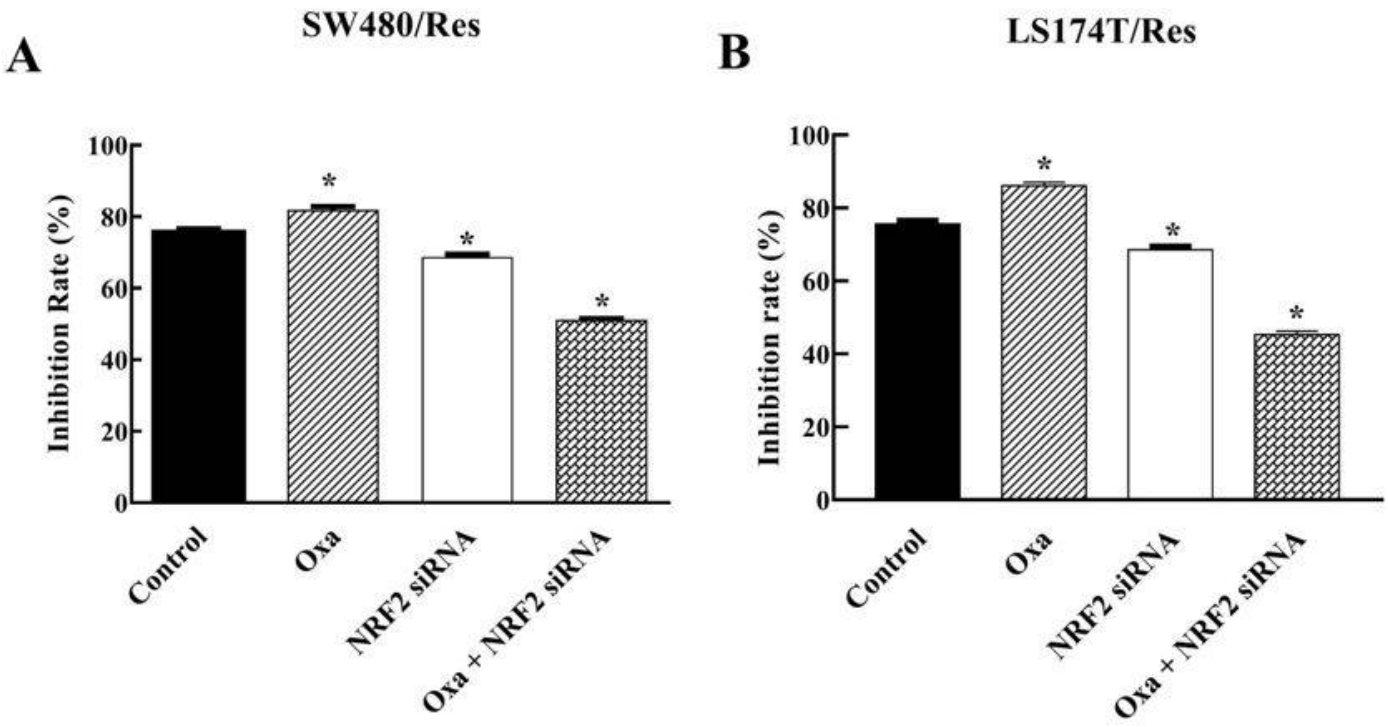


Figure 10

The impact of Nrf2 siRNA on SOD activity in resistant colon cells. SW480 and LS174T resistant (Res) colon cancer cells were transfected with Nrf2 siRNA (Nrf2 si) (80 pmol). Then, SOD activity was assessed by using superoxide dismutase activity assay. Inhibited activity of SOD in SW480/Res (A) and LS174T/Res (B) cells. The results were gathered at least from three independent experiments and finally expressed as mean ± SD. *$p < 0.05$.