Transcriptional Regulation of the Colorectal Cancer Stem Cell Markers, *Nanog and Oct4,* Induced by a Thermodynamic-Based Therapy Approach

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Abstract

Background: Cancer stem cells (CSC), as responsible issues to cancer development and progression, play a crucial role in tumorigenesis, recurrence, metastasis, and chemoresistance. Both hyperthermia and photodynamic therapy (PDT) may be effective for cancer treatment, particularly when combined with other therapeutic approaches. This study aimed to evaluate the effect of hyperthermia combined with PDT on colorectal CSC and the gene expression of the CSC markers, presenting a more effective approach for cancer therapy.

Methods: The study was conducted in the Pasteur institute of Iran, Tehran, Iran in 2018. We evaluated the anticancer role of hyperthermia, Gold nanoparticles coated with curcumin (Cur-GNPs) in PDT and combination of the two approaches on cell viability and the expression of CSC markers, *Nanog* and *Oct4* in colorectal cancer cell line HT-29. The cytotoxicity effect of Cur-GNPs against the cells was assessed **in vitro**. The cell viability was assessed using MTT assay, and the expression analysis of the CSC genes was evaluated using a q-real-time PCR.

Results: Cell viability was decreased by PDT ($P=0.015$) and the combination therapy ($P=0.006$) but not by hyperthermia alone ($P=0.4$), compared to control. Also, the expression of CSC markers, *Nanog* and *Oct4* was shown to significantly down-regulate in all hyperthermia, PDT and combination groups.

Conclusion: Hyperthermia combined with PDT was indicated to be more efficient in eliminating tumors than hyperthermia or PDT alone.

Keywords: Colorectal cancer stem cell; *Nanog*; Hyperthermia; Photodynamic therapy

Introduction

Colorectal cancer (CRC) is the third most commonly detected cancer with 1.8 million cases worldwide in 2018 (1-3). Currently, conventional treatments for CRC include tumor surgery, radia-

tion therapy, and chemotherapy (4). Despite advances in the therapeutics approaches, CRC has shown be resistant to these treatments alone (5). A subpopulation of CRC cells, named cancer stem cells (CSCs), contribute to resistance to radiation and chemotherapy and therefore, lead to failure of the cancer treatments (6-8). Colorectal CSCs (CCSCs) have the capacity of tumorigenesis, recurrence and metastasis (9). To overcome the drawbacks of conventional therapies, several studies have investigated the novel and combined strategies, including combination of ionizing radiation, hyperthermia and photodynamic therapy (PDT) (10-12).

Hyperthermia (also commonly known as thermal therapy or thermotherapy) occurs when an individual's body temperature (local, regional or whole body) increases (13). Hyperthermia has been found to be highly effective in CRC therapy specially when combined with chemotherapy, radiotherapy, or immunotherapy (14, 15). Mild hypothermia or fever-range hyperthermia had inhibitory effect on viability/proliferation in glioblastoma, prostate, lung and breast cancer cell lines (16). However, hyperthermia alone had no significant effect on breast cancer cells but its combination with radiotherapy might be useful through increasing apoptosis (17). Hence, we need to clear the possible discrepancy in our knowledge on anticancer effects of hyperthermia. Cancer therapy with light (i.e. PDT) is a non-invasive strategy that can also be effective as a supporting treatment for various types of cancer and malignant tumors (18). It use a photosensitizer which becomes activated by laser in a specific wavelength and reacts with oxygen to generate reactive oxidant species (ROS) in target tissues, leading to cell death (19, 20). ROS can unbalance redox system as well as damage DNA and protein. Moreover, PDT can dysregulate mitochondrial activity, autophagy, and CSCs dormancy (21). One of the most studied photosensitizer is curcumin (Cur) which has numerous therapeutic properties. An increasing body of evidence suggests that Cur might an appropriate photosensitizer for PDT (22). Curcumin-based PDT cannot effect on apoptosis in melanoma (23). To deliver the photosensitizer to the malignant tumor, gold nanoparticles (GNPs) have been widely used due to their biocompatibility and low toxicity (24).

We aimed to investigate whether hyperthermia and Cur-GNPs-mediated PDT alone and in combination could effect on the cell viability and the expression of cancer stem cell markers in CRC.

Materials and Methods

In vitro cytotoxicity evaluation of Cur-GNPs

The study was conducted in the Pasteur institute of Iran, Tehran, Iran in 2018. Synthesized and characterized gold nanoparticles (GNP) coated with Cur (Cur-GNPs) (25) were generously gifted by Seyed Mohammad Amini (Radiation Biology Research Center, Iran University of Medical Sciences, Tehran, Iran). The colon carcinoma cell line (HT-29) was purchased from National Bank of Pasteur Institute (Iran) and cultured in RPMI-1640 cell growth medium supplemented with penicillin (100U/ml), streptomycin (100 μg/ml), and 10% FBS. A standard humidified atmosphere (95% air & 5% CO2 at 37 °C) was provided for continuous cell culture.

In order to evaluate Cur-GNPs cytotoxicity, 10^4 cells/well were seeded into each well of a 96-well plate. After 24h, culture medium containing a definite concentration of Cur-GNPs was added and the cells were incubated for an additional 24h. Using the same procedure, Cur dark cytotoxicity was examined by 1h incubation. For photodynamic studies, we added fresh culture medium containing Cur-GNPs (128mg/ml) and Cur (6.4μg/ml) to cells, and they were incubated for 1h. Then, the culture medium was replaced and exposed to the 150mW (15.7mW/cm^2) laser (Thor International Ltd, Amersham, Bucks, UK) for 2 min. The quantitative real-time PCR (qPCR) and MTT tests were completed after 24 h incubation.

In vitro treatment with hyperthermia, PDT and combination treatment

The cell line was cultured in RPMI-1640 supplemented with 10% of fetal bovine serum (FBS)

(Gibco, USA) and incubated at 37 °C in 5% CO_2. The medium was replaced every 2 d until the cells reached 80%-90% confluence. The cells were incubated in culture medium supplemented with 10% FBS in an incubator preheated to 42 and 43 ºC for 2 h. Control groups were incubated at 37 ºC for 2 h, as well. After hyperthermia treatment, the cells were incubated at 37 ºC for 2 h prior to analysis.

For PDT treatment, first the cells were seeded in 96-well plates and incubated with 5 µM solution of Cur-GNPs for 24 h. Cur-GNPs culture medium was then removed and the plate was washed with 350 µL PBS per well. Irradiation was accomplished at room temperature with a LED-based illumination device (PDT EDL-1; Hamamatsu Photonics K.K., Hamamatsu, Japan). The expriments were adjusted by low-power laser (32 mW 630 nm diode laser, 0.5 J/cm2, continuous mode for 360 sec). Following irradiation, fresh culture medium lacking phenol red was added, cells were incubated at 37 °C for 24 h. After the irradiation, the cells were enriched by fresh medium and incubated for 24 h. Then, cell viability was evaluated by MTT assay.

For combination therapy with both hyperthermia and PDT, a cell group was simultaneously exposed to hyperthermia and PDT with the aforementioned conditions. In order to minimize interference with the optical sensitizer and maximize the effect of the laser, the cell culture medium was enriched with low FBS (% 1). The preheated cells at 42 and 43 ºC for 2 h, were cultured in the presence of Cur-GNPs and treated with low-power laser. The cells with no treatment were considered as control in all experiments. Then, MTT test was performed to evaluate cell viability.

Cell morphology

To evaluate the effect of the hyperthermia and PDT on the cellular phenotype, the cells were evaluated using an inverted microscope.

Cell viability assay

After treatments, the cell viability was determined by MTT [3-(4, 5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide] (BioIDEA,Iran)

assay. For MTT assay, HT-29 cells were seeded in 96-well plates a day prior to the experiment. The cell viability was evaluated after hyperthermia exposure. Then, the culture medium was aspirated and 10µl MTT solution with a final concentration of 0.5 mg/mL was added. Afterward, 3 h of incubation at 37 °C, MTT solution was aspirated and 100µl well DMSO was added to each well. After 30 min incubation at 37 °C, the absorbance ($^{570}/_{630}$ nm) was measured using a microplate reader (BioTek, USA).

RNA extraction and cDNA synthesis

Total RNA was extracted from cells using high pure RNA isolation kit (Roche, Germany) according to the manufacturer's instructions. The concentration of RNA was quantified using Nano Drop™ Lite Spectrophotometer (Thermo Fisher Scientific, USA). A 2% agarose gel electrophoresis was used to assess quality of RNA. Subsequently, cDNA was synthesized from the purified total RNA using a PrimeScript RT reagent Kit (Takara, Japan) according to the manufacturer's protocol.

Primer design for qPCR

The GeneRunner software was used to design the primers for amplification of interested genes. Additionally, the primer specificity was confirmed by Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast). The sequences of primers used in the current study are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as internal control for normalization (Table 1).

Quantitative Real-Time PCR

To analyse mRNA levels, qPCR was performed using SYBR® Premix Ex Taq™ II (Takara, Japan) and the ABI7500 system (Applied Biosystems; Thermo Fisher Scientific). The reaction system included 10 *µ*LSYBR_ *Premix Ex Taq*_II (2x), 0.4 *µ*L ROX dye, 2 *µ*L template cDNA, 0.4 *µ*L each primer, and 6.8 *µ*L RNase-free water. The qPCR reactions were performed in the following cycling conditions: The initial denatura-

tion step 95 °C for 30 sec, then 40 cycles of 95 °C for 5 sec and 60 °C for 30 sec. All experiments were repeated at least three times.

Table 1: Primers designed for amplification of interested gene

DNA name	fragment	Primer name	Primer sequence	Product length
OCT4		F	5´ CTTGAATCCCGAATGGAAAGGG 3´	164
		R	5´ GTGTATATCCCAGGGTGATCCTC 3´	
NANOG		F	5´ TTTGTGGGCCTGAAGAAAACT 3´	116
		R	5´ AGGGCTGTCCTGAATAAGCAG 3´	
GAPDH		F	5´ CACCAGGGCTGCTTTTAAC 3´	190
		R	5´ ATCTCGCTCCTGGAAGAT 3´	

Statistical analysis

The SPSS 19.0 (IBM Corp., Armonk, NY, USA) software was used for statistical analysis. Kruskal Wallis test was used to compare the experiments. Gene expression data derived from qPCR were analysed using GraphPad Prism 7.0 software. Data were presented as mean ± SEM. The P-values <0.05 were considered statistically significant.

Results

Cell morphology assay

The inverted microscope results showed that the hyperthermia and PDT had no effect on cell morphology.

Hyperthermia decreased expression of CSC marker but did not affect cell viability

Hyperthermia did not decrease cell viability of HT-29 cells compared to the control (37 °C) (Fig. 1a). However, expression of *Nanog* gene was significantly decreased in the HT-29 cells treated with hyperthermia at 42 °C ($P=0.038$), but not at 43 °C ($P>0.05$). Additionally, there was no significant difference in *Oct4* gene expression between hyperthermia group and the control (Fig. 1b).
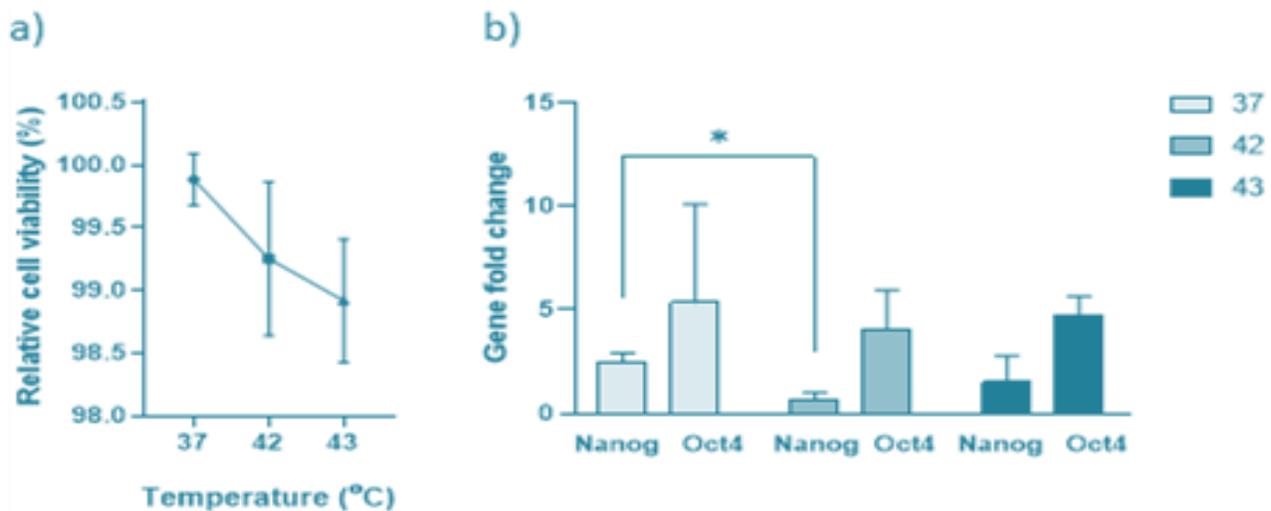
Fig. 1: a) Cell viability after treatment with hyperthermia. **b)** Effect of hyperthermia on expression of *Nanog* and *Oct4* measured by qPCR in HT-29 cell line. *$P<0.05$

PDT decreased cancer cell viability and downregulated expression of CSC markers

The cell viability of HT-29 was significantly decreased by Cur-GNPs-mediated PDT in a dose-dependent manner (Fig. 2a). *Nanog* and *Oct4* gene expression also decreased ($P<0.05$) (Fig. 2b).
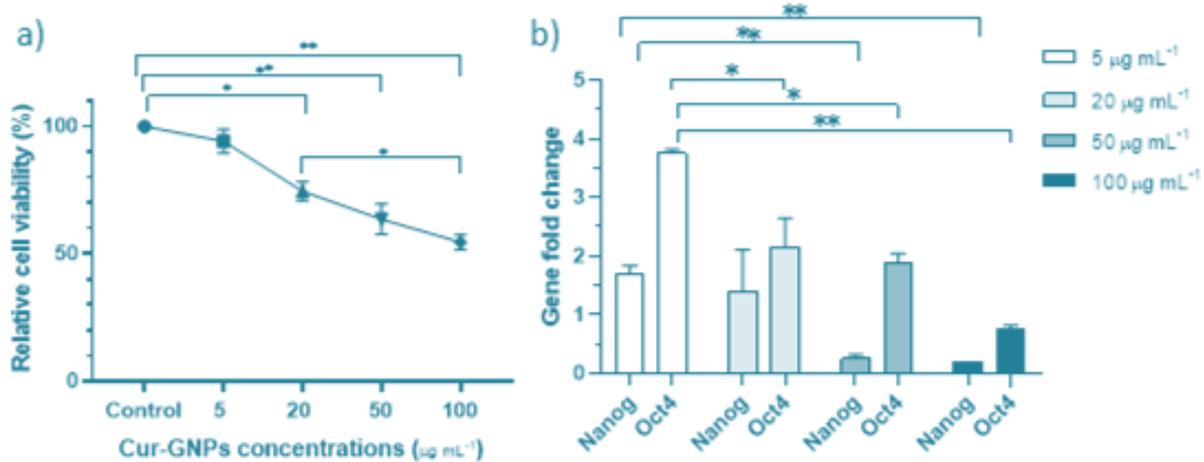

Fig. 2: a) Cell viability after treatment with PDT. **b)** Relative gene expression of *Nanog* and *Oct4* induced by PDT with various concentrations of Cur-GNPs. *$P<0.05$, **$P<0.01$, ***$P<0.001$

Combination of PDT and hyperthermia was more effective in downregulating gene expression of CSC markers

Combination of hyperthemia and PDT was more effective in reducing cell viability than hyperthermia or PDT alone (compared to hyperthermia; $P<0.01$, compared to PDT; $P<0.05$), (Fig. 3a). Moreover, the combination of PDT and hyperthermia downregulated the gene expression of *Nanog* and *Oct4* ($P<0.05$) (Fig. 3b).
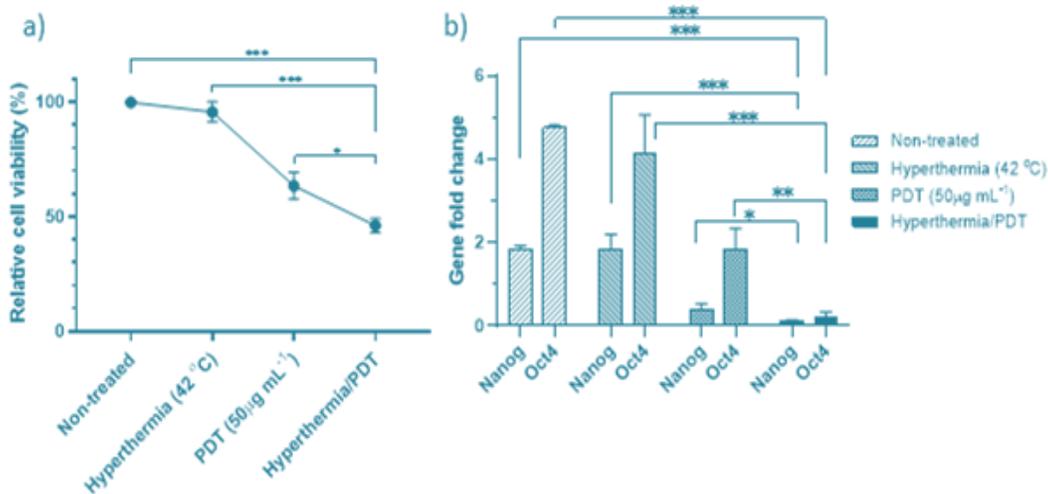

Fig. 3: a) Cell viability after treatment with a combination of PDT and hyperthermia **b)** Relative effect of PDT and hyperthermia combination therapy on gene expression of *Nanog* and *Oct4*. *$P<0.05$, **$P<0.01$, ***$P<0.001$

Discussion

Given the anticancer effects of hyperthermia and PDT, the present study aimed to investigate whether hyperthermia and Cur-GNPs-mediated PDT alone and in combination could effect on the cell viability and the expression of CCSC markers. Based on our findings, PDT but not hyperthermia, was effective in reducing cell viability. Both treatments significantly reduced gene expression of CCSCs markers. However, combination of hyperthermia and PDT was more effective than hyperthermia and PDT alone.

Hyperthermia exposure downregulated the stemness-related genes such as *Abcg2* and *Nanog* in colon cancer cell lines (26). Hyperthermia might contribute to chemosensitization of CRC. In this regard, hyperthermia has been shown to enhance the effectiveness of chemotherapy and radiation treatment (27). The hyperthermia reduced the number of CSCs cells and downregulated expression of CSC markers such as *Nanog* (6, 26). We found similar results in our study where hyperthermia significantly reduced expression of *Nanog,* a CCSC marker, but not change cell viability. Although evidence has suggested the effectiveness of hyperthermia in cancer treatment, temperature elevation alone cannot remove cancer cells (28). On the other hand, hyperthermia can inhibit repair of chemotherapy- or radiotherapy-induced DNA damage (29) and activate extracellular heat shock proteins (HSPs) leading to the antitumor immune system from the heat-treated necrotic tumor cells (30). Thereby, a series of events have been revealed to cause cell death. After mild hyperthermia, some cellular functions can be recovered and some subpopulations of cells may be resistant to hyperthermic-induced cell death (15). Thus, inability to reduce cell viability may be due to the rate of temperature or short time hyperthermia exposure.

Furthermore, we used PDT to treat colorectal cancer cell HT-29. We found that the expression of CCSC markers as well as the cancer cell viability were significantly decreased by PDT. This finding was consistent with previous studies who reported a beneficial role for PDT in treating several types of cancer, including glioblastomas, lung, esophageal, bladder, colorectal and nasopharyngeal carcinomas (31). In this regard, PDT-generated ROS could change the activity of CSC markers *Oct4* and *Sox2* (21). Furthermore, ROS might attack DNA causing point mutations in key genes such as *Ras* (32) and *p53* (33) which regulate several cellular processes, including regulate proliferation, differentiation and apoptosis (34). In addition, ROS binds to lipids and produces free radicals and peroxides that can damage cell membranes and change mitochondrial permeability leading to apoptosis (35).

According to other investigations, PDT combined by other methods could improve some cancer treatments. The effects of PBMT (Photobiomodulation therapy) combined with PDT have investigated in head and neck cancer (HNC) cell lines. Combination therapy of Photobiomodulation (PBM) and PDT functioned in some HNC cell lines such as SCC-25 cells by increasing PS uptake and production of ROS. However, the potential anticancer effect was not seen in SCC-4 cells compared to the PDT only group. PBM also improved cell metabolism by increasing uptake and oxygenation the reaction site, promoting PDT efficiency (36). A natural anthraquinone called Emodin, has cytotoxicity effects and anti-tumor activity in PDT. Moreover, emodin combined with PDT in cervical carcinoma cell lines was effective. The significant decrease in cell viability induced by the combination therapy (emodin-PDT) was due to apoptosis and autophagy mediated by ROS production in tumor cells (37).

Here, we used a combination of hyperthermia, and PDT. Cell viability and CCSC marker expression were significantly lower compared to hyperthermia, PDT or the control. Previous studies have also reported a decrease in cell viability and increased DNA fragmentation and ROS production (38, 39). The combination therapy induced the mitochondrial activation and increased the *Caspases-9, -3* and poly ADP-ribose polymerase (*PARP*) expression leading to apoptosis (40).

Hyperthermia increased efficacy of PDT on gastric cancer cells through elevated ROS production leading to enhanced PDT-induced cytotoxicity (38). The increased treatment efficiency in the combination therapy might be due to down-regulation of proliferation-related genes, increased ROS production and mitochondrial-dependent apoptosis (41). Our study showed the anti-cancer effects of combined hyperthermia and PDT in vitro system. Although, in vitro studies primarily support clinical applications, for prospective studies, it is suggested more investigation on several CRC cell lines, using various irradiation dose and animal models. In brief, we provided an important evidence of the effectiveness of hyperthermia combined with PDT in decreasing cell viability via regulating stemness-related pathways in CRC. However, the underlying molecular mechanisms of the combination of hyperthermia and PDT is still unknown. Future studies should focus on understanding the mechanism of cancer treatment via possible combination therapies.

Conclusion

Our results suggest that PDT in combination with hyperthermia may be more effective in treating and eliminate primary and aggressive colorectal tumors by affecting on cancer stem cells.

Journalism Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

Conflict of interest

Authors declare no conflict of interests.

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