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Original Article

Gene Expression Patterns of Colorectal Cancer Stem Cells Following Ibuprofen and Hyperthermia Treatment

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Abstract

Background: Cancer stem cells (CSCs) substantially influence the development of colorectal cancer (CRC), metastasis, relapse, and resistance to therapy. Ibuprofen and hyperthermia can be effective in the treatment of cancer. Herein, we evaluated the effects of hyperthermia and ibuprofen on the isolated-CSCs of CRC.

Methods: This experimental study was conducted between Sep 2020 and Jan 2022 at the Department of Pathology, School of Medicine, Shiraz University of Medical Sciences, Iran. A non-adhesive culture system was used to isolate CSCs from HT-29 cells. To confirm the stemness nature of isolated-CSCs, the expression of stemness genes and protein markers was evaluated by quantitative Real-time PCR (qRT-PCR) and flow cytometry assay. The isolated-CSCs were treated with hyperthermia and ibuprofen. The cell viability was determined by MTT assay and trypan blue staining. The expression of stemness, proliferation, Wnt signaling pathway and apoptosis genes was assessed by qRT-PCR.

Results: CSCs were isolated within 14 days. The expression of CD-133 marker and *OCT3/4*, *C-MYC*, *KLF4*, and *NANOG* genes in isolated-CSCs was higher than HT-29 cells ($P<0.05$). Cell viability of treated-CSCs were considerably reduced ($P<0.05$). Hperthermia reduced the expression of *OCT3/4*, *NANOG*, *PCNA*, *WNT1* and *CTNNB1* genes and increased the expression of *P53*, *BAX*, and *KLF4* genes ($P<0.05$). Ibuprofen decreased the expression of *OCT3/4*, *BCL2*, *NANOG*, *PCNA*, *WNT1*, and *CTNNB1* genes and increased the expression of *P53*, *BAX*, and *KLF4* genes in treated-CSCs ($P<0.05$).

Conclusion: Hyperthermia and ibuprofen treatment demonstrate an inhibitory effect on colorectal CSCs. However, using combination therapy is remaining to be tested.

Keywords: Colorectal cancer; Hyperthermia; Ibuprofen; Cancer stem cell

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed gastrointestinal malignancies after skin, breast and stomach cancer, and the fourth most common reason of mortality caused by cancer globally (1). Molecular changes in the epithelial cells of the colon or rectum cause

CRC, which originates from mutations in oncogenes, tumor suppressor genes, and DNA repair genes. (2). The incidence of CRC is considerably increasing due to lifestyle changes, decreased physical activity, smoking and poor diet (3-5). At present, the most effective modalities to treat CRC include surgery, chemotherapy and radiotherapy. Despite advances in these treatment methods, the survival rate of cancer patients is limited and recurrence of the disease is often observed after treatment (6). Besides, each of the various current drugs used for chemotherapy has severe side effects, the main ones are neuropathy and neutropenia (7).

One of the biggest problems in cancer treatment is drug resistance (8). Therapy failures are often caused by mutations in the key molecules of signaling pathway, an increase in anti-apoptotic proteins, the existence of tumor stem cells that are resistant to chemotherapy, and the overactivation of drug efflux pumps (9).

The cancer stem cells a subset of cancer cells called cancer stem cells (CSCs) play an essential role in the initiation and growth of various types of cancer, such as CRC (10). As with normal stem cells, CSCs exhibit characteristics such as self-renewal, asymmetric cell division, quiescence, multipotency, and expression of drug and apoptosis resistance genes (11, 12). The presence of CSCs has been detected in a wide range of solid tumors, including the brain, breast, lung, prostate, and CRC (13). Conventional modalities used to treat cancers, frequently aim for the bulk of the tumor but fail to eradicate the CSCs because of their strong resistance (14). Hence, CSCs are responsible for high cancer therapeutic failure rates, and it is crucial to find new therapy modalities, particularly those, which inhibit and treat CSCs.

Hyperthermia is a cancer treatment modality, which raises the temperature of a part of or the whole body above normal (37°C). The increasing temperature of 41 °C to 47 °C induces cell death via apoptosis while increasing temperature above 50 °C lead to cell necrosis (15). The main mechanism of hyperthermia is destructing proteins and the structure within cells (16). Treatment of cancer cells with hyperthermia is applied alone or in combination with various established cancer treatment modalities such as radiotherapy and chemotherapy (17). The use of hyperthermia in combination with chemotherapy increases the cytotoxicity of chemotherapy agents via enhancing endothelial cell permeability and local blood flow (16).

In addition, certain cancers including prostate, colon, breast, lung, and gastric can be prevented with non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen (18). Furthermore, ibuprofen induced apoptosis and inhibited cell proliferation in human cancer cell line (19).

Accordingly, in the present study, first we isolated and characterized CSCs from colorectal cancer cell line (HT-29). The expression of stemness, proliferation, apoptosis and Wnt signaling pathway genes was then examined in treated CSCs with hyperthermia and ibuprofen to comprehend the molecular processes through which hyperthermia and ibuprofen slow tumor growth in colorectal cancer cells.

Materials and Methods

Cell culture and cancer stem cell isolation

The human colon cancer cell line HT-29 (Cell Number: IBRC C10097) was purchased from the Pasteur Institute Cell Bank, Tehran, Iran. This experimental study conducted at the Department of Pathology, School of Medicine, Shiraz University of Medical Sciences between Sep 2020 to Jan 2022. HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% CO2 and 95% humidity at 37 °C (20). Cells were subcultured at 70% confluence following treatment with 0.25% Trypsin–EDTA (Sigma-Aldrich, USA). All experiments were performed with mycoplasma-free cells within 3 months from the purchase of the cell line. Then, sphere formation assay was used to isolate CSCs from parental HT-29 cells as described previously (21).

Evaluation of CSCs properties

To elucidate whether isolated-CSCs could enrich the expression of stem cell markers, we analyzed

CD-133 expression using flow cytometry and *OCT3/4*, *C-MYC*, *NANOG*, and *KLF4* gene expression using quantitative Real-time PCR (qRT-PCR). Phosphate buffer saline (PBS) was used to wash the cells prior to fluorescence-activated cell sorting (FACS). Then, single-cell suspensions in PBS were incubated with antibodies against CD-133 (SANTA CRUZ BIOTECHNOLOGY, INC.). After 25 min of incubation on ice, the labeled cells were washed and centrifuged at 2100 g for 5 min. The samples were resuspended in PBS and analyzed by flow cytometer (BD FACSCalibur system) (21). HT-29 cell lines were used as controls and all other data were compared to the controls.

Hyperthermia and ibuprofen treatment

To evaluate the effects of hyperthermia and ibuprofen on CSCs, 3×10^4 cells were seeded into 24-well plate with DMEM containing 10% FBS at 37 °C and 5% CO2. Before hyperthermia treatment, the medium was replaced with a fresh culture medium. Then, hyperthermia was applied at 42 and 43 °C for 3 h in a culture cabinet with 5% CO2 and 95% humidity. Control cells were exposed at 37 °C with 5% CO2 and 95% humidity. A fresh DMEM medium supplement with 3% FBS and different concentrations of ibuprofen (700, 900, 1100, 1300 and 1500 µM) was used for ibuprofen treatment. Control groups included untreated CSCs. All the experiments were carried out in triplicate.

Cytotoxicity and cell viability

The effects of hyperthermia and ibuprofen on the cell viability of CSCs have been studied using the MTT assay. Briefly, CSCs ($2x10^4$ cells/well) were seeded in a 96-well plate and treated with hyperthermia and various concentrations of ibuprofen at 24 and 48 h as previously described (22). The untreated CSCs were considered as a control group. By comparing the optical density (OD) of treated CSCs with untreated CSCs, the degree of hyperthermia and ibuprofen cytotoxicity was quantified. Additionally, dose-response curves were used to assess the 50% inhibitory concentration values (IC50) of ibuprofen against CSCs.

CSCs growth curve

To evaluate the effects of hyperthermia and ibuprofen on CSCs, trypan blue staining and cell counting were performed. CSCs (3×10^4 cells/well) were cultured in 24-well plates and treated with hyperthermia and ibuprofen. The untreated-CSCs were considered as a control group. After three different incubation times (24, 48, and 72 h), cells were detached by 0.25% trypsin-EDTA (Sigma, USA) and stained with 0.4% trypan blue dye (Sigma, USA).

RNA extraction and qRT-PCR

The effect of hyperthermia and ibuprofen on the expression of stemness-related genes (*OCT3/4*, *NANOG*, and *KLF4*), proliferation (*PCNA*), Wnt signaling pathway (*WNT1* and *CTNNB1*), and apoptosis pathway (*P53*, *BAX*, and *BCL2*) were examined by qRT-PCR. After hyperthermia and ibuprofen treatment, the cells were trypsinized and total RNA was extracted using the RNeasy Mini kit (Qiagen, USA) in accordance with the manufacturer's instructions. RNA extraction was also performed from untreated CSCs as a control group. A nanodrop (Thermo Fisher, USA) was used to measure the concentration of the extracted RNA, subsequently kept at -80 °C until use. Next, using the NG dART RT kit (EURX, Poland), complementary DNA (cDNA) was produced from total RNA according to the manufacturer's instructions.

Primer sequences were designed using GeneRunner and AlleleID software (Table 1). In addition, Primer-BLAST was used to confirm primer specificity (www.ncbi.nlm.nih.gov/tools/primer-blast). The *GAPDH* gene (*Glyceraldehyde-3-phosphate dehydrogenase*) was used as a housekeeping gene. Finally, quantitative gene expression was performed by SYBRGreen PCR kit (Takara, Japan) and on ABI step one plus Real-time PCR System.

Table 1: Primer sequences used in qRT-PCR

Name	*Primer name*	*Primer sequence*	*Tm (°C) X**	*Product length*
OCT4	OF	5′GAGAACCGAGTGAGAGGCAACC3′	68	166
	OR	5′CATAGTCGCTGCTTGATCGCTTG3′		
NANOG	NF	5′GTCCCGGTCAAGAAACAGAAG3′	58	156
	NR	5′GTCTTCACCTGTTTGTAGCTG3′		
KLF4	KF	5′GTGCCCCGAATAACCGCTG3′	62	203
	RF	5′CAGGTCCAGGAGATCGTTGAAC3′		
C-MYC	MF	5′CACTTTGCACTGGAACTTACAACACCC3′	64	183
	MR	5GCGGGAGGCTGCTGGTTTTC′3′		
WNT1	DF	5′CGATGGTGGGGTATTGTGAAC3′	60	133
	DR	5′CCGGATTTTGGCGTATCAGAC3′		
CTNNB1	LF	5′ACGTACAATAGCAGACACCATC3′	60	146
	LR	5″TCAGGGAGTCAGGGGAGG3′		
P53	SF	5″TAACAGTTCCTGCATGGGCGGC3′	66	121
	SR	5′AGGACAGGCACAAACACGCACC3′		
BAX	GF	5′CCTGTGCACCAAGGTGCCGGAACT3′	68	99
	GR	5′CCACCCTGGTCTTGGATCCAGCCC3′		
BCL2	BF	5″TTGTGGCCTTCTTTGAGTTCGGTG3′	64	114
	BR	5′GGTGCCGGTTCAGGTACTCAGTCA3′		
PCNA	CF	5″TAACAGTTCCTGCATGGGCGGC3′	60	126
	CR	5′CGTGCAAATTCACCAGAAGGC3′		
GAPDH	GF	5′GGACTCATGACCACAGTCCA3′	60	119
	GR	5′CCAGTAGAGGCAGGGATGAT3′		

Key: The annealing temperature for each gene is shown by X*

Statistical analysis

All experiments were repeated at least three times. The SPSS ver. 22 (IBM Corp., Armonk, NY, USA) software was used for data analysis. To determine whether there were statistically significant differences between the experiments, one-way analysis of variance (ANOVA) and Tukey's test were used. In addition, qualitative control of qRT-PCR results, changes in gene expression, and statistical analysis were performed by GenEX software. The *P*-values less than 0.05 were considered statistically significant. Data were presented as mean ± SEM.

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

Results

Isolation and characterization of CSCs

HT-29 cells were adherent cells with epithelial morphology and had a high growth rate. After three days, the cells reached 70% confluency and were sub cultured (Fig. 1, A). CSCs isolation from HT-29 cells was performed via a sphere formation assay in non-adherent conditions on agar-coated plates. During the first four days, part of the cell suspension was subjected to apoptosis. HT-29 cells formed spheres with a round shape, smooth surface, and compact morphology during 10 to 14 days post culture. The spheres were darker in the center and lighter at the edges (Fig. 1, B).

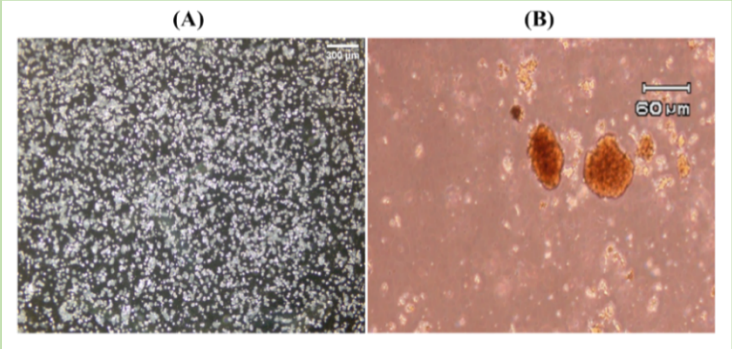

Fig. 1: **(A):** The monolayer HT-29 cell culture (Scale bar=300 µm) and **(B):** Isolated-CSCs from HT-29 cells (Scale bar= 60 µm)

For the characterization of isolated-CSCs, the expression of the CD-133 surface marker was examined by flow cytometry. As shown in Fig. 2(A), approximately 92% of isolated-CSCs were positive for the CD-133 marker (*P*=0.008). In addition, qRT-qPCR was used to analyze the gene expression of four stem markers including *OCT3*/4 (*P*=0.006), *SOX2(P*=0.02, *KLF4 (P*=0.001) and *C-MYC* (*P*=0.001) in HT-29 cell lines and isolated-CSCs. As shown in Fig. 2 (B), the expression of all four Stem cell markers in isolated-CSCs was significantly higher than HT-29 cells.

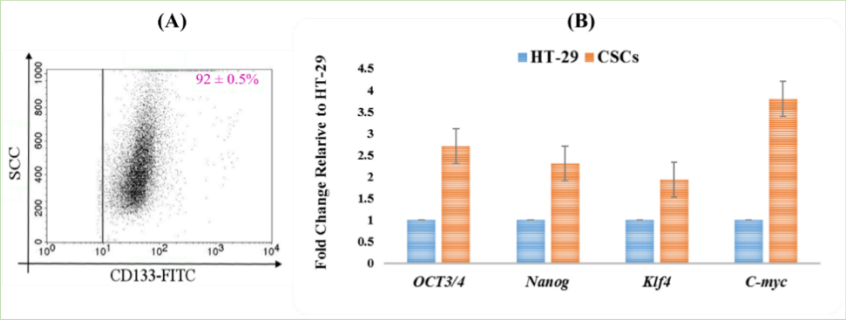

Fig. 2: **(A):** CD-133 expression analysis by flow cytometry. CD surface marker was significantly presented in isolated-CSCs compared with HT-29 cells. **(B)** qRT-PCR analysis: the expression of four stemness genes was significantly increased in the isolated-CSCs compared with HT-29 cell lines

Toxicity and cell viability

The effects of hyperthermia and ibuprofen on the cell viability of CSCs were evaluated using MTT assay at 24 and 48 h. Cell viability of untreated-CSCs was above 92% for all groups. The viability of hyperthermia treated-CSCs at 42 and 43 °C was meaningfully lower than untreated-CSCs (*P*=0.0001). Furthermore, the viability of ibuprofen treated-CSCs reduced in concentration-dependently. As shown in Fig. 3, no statistically significant difference was found between treated and untreated CSCs after 24 or 48 h in any group (700, 900, or 1100 µM). However, the cell viability of CSCs treated with ibuprofen (1300 and 1500 µM) was significantly reduced (*P*=0.0001). The IC50 value of ibuprofen in treated-CSCs at 24 and 48 h were 1210 µM and 1190 µM, respectively. Therefore, the gene expression was evaluated with ibuprofen at concentration 1100 µM.

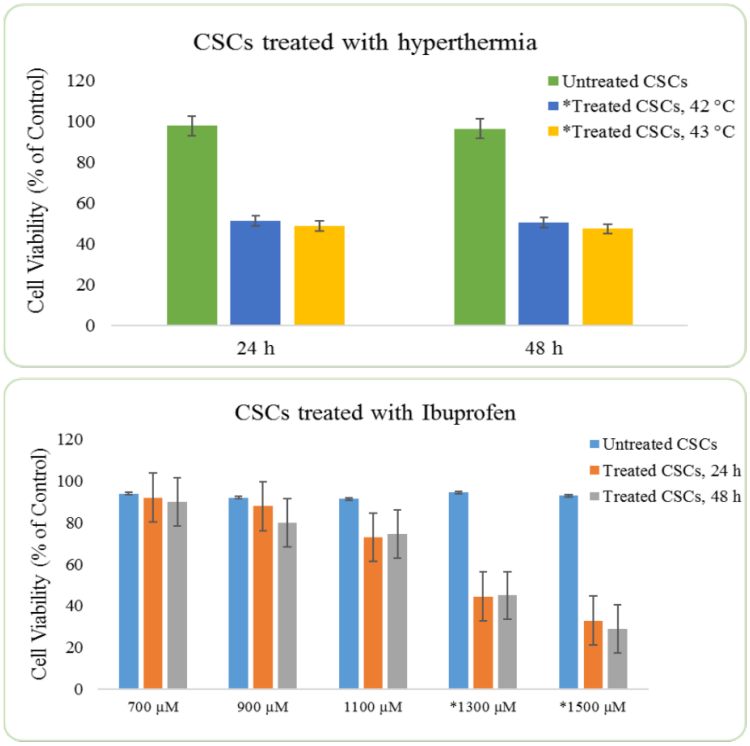

Fig. 3: Cell viability of treated-CSCs with hyperthermia and ibuprofen. The star (*) attached to the curve indicates $P< 0.05$

CSCs growth curve

The effects of hyperthermia and ibuprofen on the proliferation were evaluated using trypan blue staining and cell counting at 0, 24, 48, and 72 h. As shown in Fig. 4, the proliferation of hyperthermia treated-CSCs (42 and 43°C) was significantly decreased after 24 h compared with untreated-CSCs (P=0.0001). In addition, the proliferation of ibuprofen treated-CSCs (700, 900, and 1100 µM) was similar to untreated-CSCs until 72 h. However, the proliferation of CSCs treated with ibuprofen (1300 and 1500 µM) was significantly reduced after 24 h (P=0.0001).

Gene Expression Study

Effects of ibuprofen and hyperthermia on a group of genes were examined by qRT-PCR assay. As shown in Fig. 5, the expression of *OCT3/4*, *NANOG*, *PCNA*, *BCL2*, *WNT1* and *CTNNB1* genes in CSCs treated with hyperthermia at 42 °C and 43 °C was lower than the untreated-CSCs. However, the expression of these genes in CSCs treated with hyperthermia was not significantly different from each other, except for the *BCL2* gene (P<0.05). The expression of *P53, KLF4*, and *BAX* genes was increased in CSCs treated with hyperthermia in comparison with the untreated-CSCs (P-value was presented in Table 2, separately). No significant difference in gene expression was observed between the treated-CSCs at 42 °C and 43 °C. Ibuprofen at concentration 1100 µM downregulated the expression of *OCT3/4*, *NANOG*, *PCNA*, *BCL2*, *WNT1* and *CTNNB1* genes, nevertheless, upregulated the expression of *KLF4*, *P53*, and *BAX* genes in CSCs compared with untreated-CSCs.

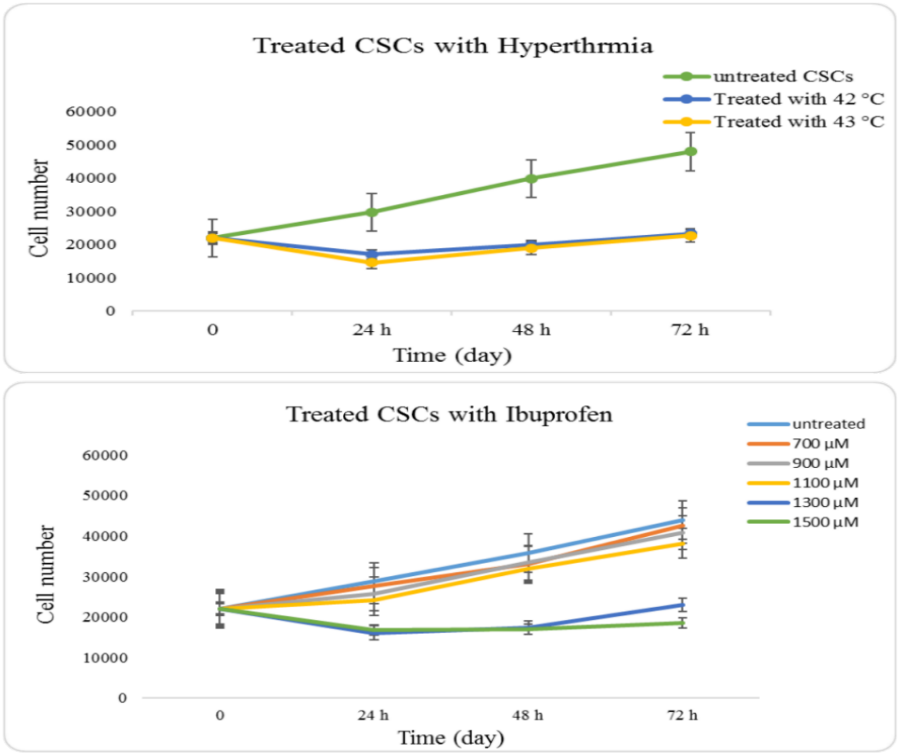

Fig. 4: Treated and untreated-CSCs growth curve

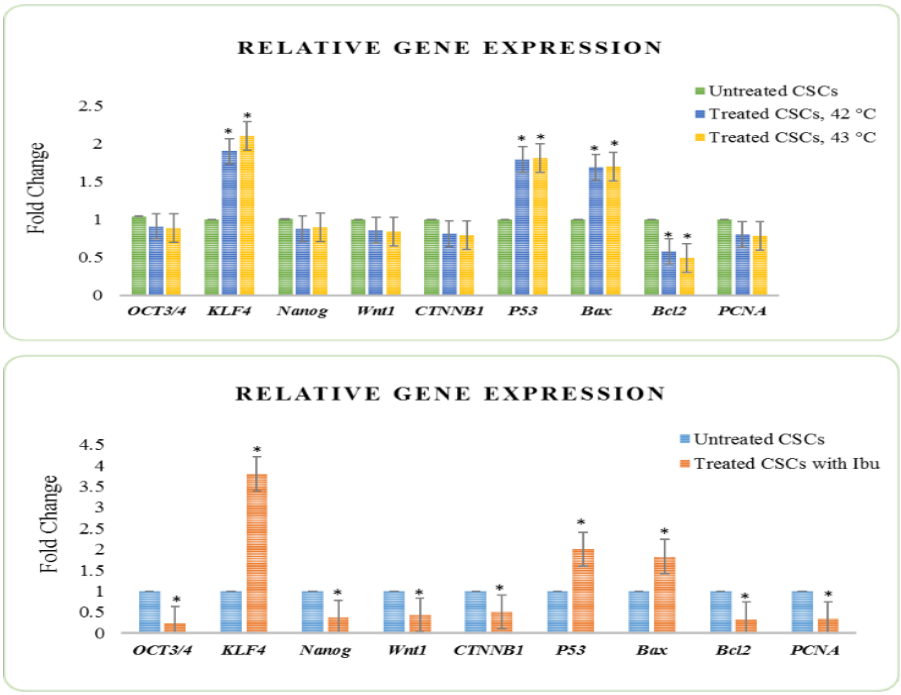

Fig. 5: Effect of hyperthermia and ibuprofen on the gene expression of CSCs. The star (*) attached to curve indicates $P<0.05$

Table 2: Effect of hyperthermia on the expression of *KLF4*, *P53*, *BAX* and *BCL2* genes

GENE	*(Mean Fold Change) at 42°C*	*SD*	*SEM*	*P-Value*	*(Mean Fold Change) at 43°C*	*SD*	*SEM*	*P-Value*
KLF4	1.52	0.036	0.021	0.0001	1.63	0.022	0.012	0.0002
P53	1.69	0.10	0.05	0.031	1.83	0.019	0.011	0.012
BAX	1.3	0.013	0.007	0.019	1.34	0.037	0.021	0.008
BCL2	0.89	0.005	0.003	0.005	0.86	0.014	0.008	0.0001

Discussion

Despite the growth being made in colorectal cancer therapy, it remains one of the most common reasons for cancer-related death worldwide (1). Cancer stem cells are responsible for the initiation of tumors, development, metastasis, relapse, and resistance to treatments in CRC (11, 23, 24). Conventional therapies, such as chemotherapy, target proliferating and mature cancer cells. Nevertheless, CSCs escape from conventional therapies by continuing dormant, reducing cell cycle speed, high DNA repair capacity, turning off apoptotic pathways, epithelial-mesenchymal transition (EMT), over-expression of anti-apoptotic proteins, upregulation of multidrug resistance membrane transporters, and manage reactive oxygen species (ROS) (14, 25). Consequently, it is necessary to identify and develop new therapies that target important molecules and signaling pathways of colorectal CSCs.

CSCs have been isolated from solid tumors using three different methods, including fluorescence-activated cell sorting (FACS) based on CSC-specific cell surface markers (26), Sorting CSCs using the intracellular Hoechst 33342 exclusion method (27), and the spheroid body formation assay (28). In the current study, we used the spheroid body formation assay in non-adherent conditions for the isolation of CSCs from HT-29 cell lines. In comparison with the prior sphere culture method, this spheroid body culture system is more cost-effective and requires no growth factors, hence it has become more widely utilized as a method for enriching CSCs (29).

In the following, we investigated the stemness properties of isolated-CSCs and we found that the stemness genes including *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC* were over-expressed in the spheres (CSCs) compared with HT-29 cells. Furthermore, we evaluated the CD-133 expression marker, and the results showed that the expression of the CD-133 marker in the spheres was significantly higher than HT-29 cells. Among the identified protein markers, CD44 and CD133 have been extensively observed in tumors with the epithelial-mesenchymal origin and were considered major markers of tumor-initiating populations in many solid tumors, such as the colon (30, 31).

Afterwards, the effects of hyperthermia and ibuprofen on viability, proliferation, and the expression of stemness-related, proliferation, Wnt/β-catenin signaling pathway, and apoptosis genes were evaluated. Our results indicated that hyperthermia and ibuprofen were significantly more effective in reducing the survival and proliferation of treated-CSCs. Ibuprofen reduced the expression of the proliferation-related gene (*PCNA*) in the treated-CSCs, which was consistent with the inhibitory effect of ibuprofen on cell proliferation. The results of this study were also in agreement with those of previous studies showing that ibuprofen inhibits proliferation of colon cancer (HT-29) and gastric adenocarcinoma cell lines (MKN-45) (32).

In addition, our results demonstrated that ibuprofen and hyperthermia reduced the expression level of Wnt/β-catenin signaling pathway genes (*WNT1* and *CTNNB1*) in the treated-CSCs. We

observed the expression of stemness genes including *OCT3/4* and *NANOG* significantly decreased in the ibuprofen treated-CSCs, as well. The Wnt/β-catenin signaling pathway is frequently dysregulated in different cancers, particularly in CRC (33, 34) and is involved in a variety of aspects of cancer including survival, angiogenesis, proliferation, EMT, migration, invasion, metastasis, and renewal of CSCs (35). Overactivation of the Wnt signaling pathway disrupts the normal growth and differentiation of colon stem cells and leads to colorectal CSC phenotype by over-expression of *c-MYC*, *Bcl-2 Associated X* (*Bax*), and *cyclin D* genes (36). Therefore, downregulation of the Wnt/β-catenin signaling pathway by crosstalk with other signaling pathways can obstruct cell proliferation and induce apoptosis in a wide range of malignant tumors.

Escape from apoptosis is one of the hallmarks of any tumor-initiating cells, such as CSCs. Many therapeutic agents perform their anti-tumor effects through the apoptosis signaling pathway (37). Hence, we also investigated the effect of hyperthermia and ibuprofen on the expression of apoptosis-related genes. We found that the expression of *P53* and *BAX* genes was significantly upregulated in the treated-CSCs and the expression of *BCL2* gene was significantly downregulated. One of the important regulators of apoptosis, cell cycle progression, and angiogenesis is the *P53* marker. Ibuprofen induces cell apoptosis and inhibits cell proliferation in HCT-116 colon carcinoma cells by increasing the expression of *P53* and *BAX* proteins (38).

Besides, our results indicated the expression of *KLF4* gene in the ibuprofen treated-CSCs was significantly higher than untreated-CSCs. *KLF4* marker has been extensively studied as a tumor suppressor or an oncogene depending on the context of tumors (39). Evidence indicated that *KLF4* protein can successfully suppress colorectal cancer cell proliferation or migration (40), which was also in consent with the results of the current study.

Conclusion

Ibuprofen and hyperthermia can lessen the tumour properties of HT-29 cells by causing apoptosis and preventing cell division. Additionally, downregulation in transcript level of stemness genes revealed that ibuprofen decreased the stemness of the HT-29 cells. Taken together, the present investigation indicates that, between hyperthermia and ibuprofen treatment, ibuprofen has significantly against human colorectal CSCs compared with hyperthermia. Therefore, we suggested that hyperthermia in combination with ibuprofen may be considered a new direction in the treatment of colon cancer and can decrease the risk of colorectal cancer.

Abbreviations

CRC: Colorectal cancer, CSC: Cancer stem cell, EMT: Epithelial-mesenchymal transition, NSAIDs: Non-steroidal anti-inflammatory drugs, DMEM: Dulbecco's modified Eagle's medium, qRT-PCR: quantitative Real-time PCR, PBS: Phosphate buffer saline, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, ROS: Reactive oxygen species, *BAX: Bcl-2 Associated X*, *KLF4*: Krüppel-like factor 4, and TME: tumour microenvironments.

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Journalism Ethics 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.

Conflict of Interest

The authors declare no competing financial interests.

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