The Effect of Quercetin and Hyperthermia on spheroid model of DU145 Prostate Carcinoma Cell line

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ABSTRACT

The purpose of this study is to investigate the effects of Quercetin as an inhibitor of heat shock proteins and hyperthermia on the induced DNA damages and colony formation ability of DU145 tumor spheroid culture. DU145 cells were cultured as spheroids. On day11, spheroids with mean diameter 100 μm were treated with different concentration of Quercetin for 24 hours and then exposed to hyperthermia at 43°C for 1 hour. After heat exposure, the colony forming ability and the induced DNA damages were examined using colonogenic and alkaline comet assay methods, respectively. Our results showed that DMSO diluent in combination with hyperthermia had no significant effect on the number of colonies and the level of DNA damages as compared to control (p>0.05). Furthermore, number of colonies decreased and DNA damages increased by increasing Quercetin concentration in combined treatment of DU145 cells with Quercetin and hyperthermia in spheroid cultures. Quercetin as an inhibitor of heat shock protein 70 production in cells exposed to hyperthermia can increase DNA damages and decrease colony numbers of the prostate cancer cells in a dose-dependent manner and there is a correlation between the increase of DNA damages and decrease of colony numbers.

Keywords: Quercetin; Hyperthermia; Colonogenicity; DNA damages; Spheroid

INTRODUCTION

Prostate cancer is the most common and second cause of cancer death among men in the United States [1]. Many kinds of treatments are applied but not successful enough to reduce mortality in patients [2]. Among these treatments hyperthermia seems to be a potent tool in combination with other modalities [3]. High temperature causes direct damage to cancerous cells and also sensitize them to other types of treatments such as chemotherapy and radiotherapy while has no effect on normal cells [4]. High temperatures and other cellular assaults such as hypoxia, virus infections and tumor necrosis factor α induce the expression of a group of proteins called heat shock proteins (HSP). HSPs are highly conserved molecular chaperons, present in both eukaryotic and prokaryotic cells [5, 6]. They have critical role in assisting other cellular proteins to attain their functional conformation, mediating their interaction, assembly and movement of transmembrane proteins [7]. Expression of HSPs is increased tremendously after stress conditions as a defense mechanism to allow cells to survive[8]. It was reported that there is a correlation between HSP70 overexpression and thermal resistance [9]. Heat kills the cells by causing protein aggregation and this event is antagonized by HSP production [10-12]. It has been shown that Quercetin, a flavonoid frequent in our daily diet with anticancer, anti-inflammatory, antioxidant, antiviral and antibacterial activity, can inhibit the synthesis of HSPs in several malignant cell lines after heat and drug treatment [13, 14]. It inhibited increase of HSP expression in cells exposed to high temperature [15, 16]. In this research the moderately aggressive prostate cancer cell line [17], DU145, in spheroid form was applied. Spheroids are cell aggregates grown from one or several cell clones which exhibit many
characteristics of tumors. They seem to be an appropriate model which mimics the architecture of solid tumors, rather than monolayer cell lines [18]. Under the same conditions of heat treatment, DU145 spheroid cultures are able to produce higher amount of HSP70 compared with monolayer cultures and this may be the reason of their thermal resistance [10]. Therefore using of Quercetin as an inhibiting factor can reduce the HSP70 expression and the thermo resistance of cells in this model of culture.

The aim of this study was to examine the effects of different concentration of Quercetin and hyperthermia on colonogenicity and induced DNA damages of DU145 tumor spheroids and finally the correlation between these two biological effects.

MATERIALS AND METHODS

Human prostate carcinoma cell line DU145 was obtained from Pastor Institute of Iran. The cell line was maintained in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Biosera), 100 u/ml of Penicillin and 100mg/ml of streptomycin ( Biosera). Cells were cultured as monolayer at a density of 10^4 cells/cm^2 in T-25 tissue culture flasks (Orange). Cultures were maintained at 37ºC in a humidified atmosphere and 5% CO_2. Cells were harvested by trypsinizing cultures with 1mM EDTA/0.25% Trypsin (w/v) in phosphate buffer saline (PBS). Spheroids were cultured using the liquid overlay technique [19]. 5x10^5 cells were seeded into 100mm culture Petri dishes coated with a thin layer of 1% agar (Bacto Agar) with 10ml of RPMI supplement with 10% FBS. The plates were incubated at 37ºC in a humidified atmosphere and 5% CO_2. Half of the culture medium was replaced with fresh culture medium twice per week. On day 11, spheroids with mean diameter 100µm were treated with 100, 300 and 500µM of Quercetin (Sigma) for 24 h. Quercetin was dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO added to the medium was 0.25% (v/v). The same concentration of DMSO was added to the control cultures of drug treatment. Before heat treatment, the medium was replaced with fresh culture medium. Hyperthermia was applied at 43ºC for 60 min in a precision water bath (Memmert) with ± 0.1ºC accuracy. Control cells were exposed to 37 ºC. After heat treatment the spheroid cells were treated with 300µl of 1mM EDTA/ 0.25% Trypsin (w/v) in PBS for 5 minutes at 37 ºC. Trypsin was neutralized by the addition of 700µl culture medium containing 10% FBS. The single cells were counted and tested for viability. Half of cells were used for colony formation ability, the other half were processed for Single Cell Gel Electrophoresis (alkaline comet assay). A suspension of treated and control single cells from spheroid culture were mixed with Trypan Blue at a 9:1 ratio. The resulting mixture was examined within 3-5 minutes under a light microscope and blue colored cells were considered dead. The ratio of unstained cells to total number of cells was reported as the viability percentage for each cell category. Treated and control single cell suspensions from spheroid cultures were seeded at a density of 3000 cells per 60mm Petri dish containing 5ml of culture medium supplemented with 10% FBS. The cells were incubated at 37ºC in a humidified atmosphere of 5% CO_2. After 9 days the colonies were fixed with 2% Formaldehyde in PBS and stained with 0.5% Crystal violet. The colonies were counted using an inverted phase microscope (Olympus-CK2) and the planting efficiency was determined. The induction of DNA damages were determined by alkaline comet assay. The alkaline comet assay in this work was a modification of the method described by Singh et al [20]. Ordinary microscope slides were coated with 1% normal melting point agarose. Approximately 10,000 cells were suspended in 100µl of 0.5% low melting point agarose. The cell suspension was rapidly pipetted onto the first agarose layer. The slides were allowed to solidify, then immersed in freshly prepared lysis buffer ( 2.5M NaCl, 100mM EDTA, 10mM Tris-base with 1% Triton X-100, pH=10) and incubated for an hour. From that point on, all the steps were performed at 4 ºC. The slides were removed from the lysis buffer and placed in a horizontal gel electrophoresis tank (Cleaver Scientific Ltd, CSL-COM20) which was filled with fresh cold denaturation buffer (300 mM NaOH, 1mM EDTA, pH=13). The slides were left in the solution for 30 min. Electrophoresis was conducted in the same denaturation buffer
for 30 min using 1V/cm voltage and a current of 300 mA. Following electrophoresis, the slides were washed in Tris buffer (0.4 M Tris-HCl, pH=7.5) to neutralize the excess alkali. Finally, the slides were stained with ethidium bromide (20 μg/ml). The individual cells or comets were viewed and photographed using a fluorescent microscope (Zeiss, Axioskop 2 plus) equipped with an ethidium bromide filter (excitation filter, 535 nm; emission filter, 610 nm) and a CCD camera (Hitachi, KP-D20BP). The photographs were analyzed by Comet Score® software. DNA damages were quantified as an increase in tail moment, the product of the amount of DNA (fluorescence) in the tail and the distance between the means of the head and tail fluorescence distributions.

A total of 100 individual cells on each slide and three slides for each sample were scored visually as belonging to one of five predefined classes according to tail length and given a value of 0, 1, 2, 3, or 4 (from no tailing, 0, to maximally tailing, 4). The total score for comets could range from 0 (all no tailing) to 400 (all maximally tailing).

\[ DD\ (au) = \frac{(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4)}{\sum n}/100 \]

Where DD (au): Arbitrary unit DNA damage score, n0-n4: number of Class 0-4 comets, Σn: total number of scored comets. Coefficients 0-4 are weighting factors for each class of comet [21, 22]. One may suspect that the visual classification may be inferior to computerized analyses, such as tail moment analysis of images captured by CCD camera. DNA damages were quantified as an increase in tail moment, the product of the amount of DNA (fluorescence) in the tail and the distance between the means of the head and tail fluorescence distributions. The results are expressed as mean values ± SEM (Standard Error of Mean), with "n" denoting the number of experiment. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Turkey’s test as the post-hoc analysis using SPSS version 12. The value of p<0.05 was considered to be significant.

RESULTS

The DU145 prostate carcinoma cell line grows as monolayer on plastic culture flasks with a population doubling time of approximately 23 hrs. These cells can survive in low population densities and form colonies with at least 50 cells within 11 days. The DU145 cells are also able to form spheroids in liquid overlay cultures. In order to evaluate the colony-forming ability of DU145 cells in spheroid model of culture, 11 days spheroids with 100 µm mean diameter were used. For this purpose, various numbers of cells from spheroid culture were harvested and used to colony forming ability as described in the method sections. The colonies were counted using an inverted phase microscope on day 11 and clonal plating efficiency (PE) was determined. Figure 1 shows the colony forming ability of various concentrations of DU145 cells from spheroid cultures. Figure 2 shows the phase contrast micrograph of a colony formed 11 days after initiation of culture.

![Figure 1](image1.png)

**Figure 1.** The colony forming ability of DU145 cells from spheroid cultures. Cells were harvested from 11 day spheroids and plated in 60 mm petri dishes at various concentrations. The colonies formed 11 days after initiation of cultures were counted. Mean ± SEM of 3 experiments.

![Figure 2](image2.png)

**Figure 2.** Phase contrast micrograph of a colony formed 11 days after initiation of culture.
Immediately after cell treatment with Quercetin and hyperthermia, spheroid cells were dispersed to single cells. They were then counted and viability was determined using the trypan blue dye exclusion assay. Figure 3 shows the effect of Quercetin and hyperthermia on the viability of DU145 cells from spheroid cultures. As can be seen, hyperthermia at 43°C in combination with Quercetin and DMSO as Quercetin diluents did not have any effect on the viability of cells in spheroid culture.

![Figure 3](image)

**Figure 3.** Effects of different concentrations of Quercetin and hyperthermia at 43°C for 1 hour on the viability of DU145 spheroid culture cells. Mean ± SEM of 3 experiments.

The cell response to hyperthermia and Quercetin in terms of colony formation was studied by applying different concentrations of Quercetin plus hyperthermia at 43°C for 1 hour. Plots of colony numbers versus Quercetin concentrations for 11 days old spheroid cultures are shown in figures 4. This figure clearly shows that DMSO diluent in combination with hyperthermia had no significant effect on the number of colonies as compared to control (p>0.05). Furthermore, this figure shows that number of colonies decreased by increasing Quercetin concentration in combined treatment of DU145 cells with Quercetin and hyperthermia in spheroid cultures. Alkaline comet assay were used for evaluation of DNA damages. Figure 5 shows the intercellular distribution of DNA migration (number of cells in the five visual comet classes) among control and treated cells with Quercetin and hyperthermia at 43°C for 1 hour respectively. We observed a significant increase in number of comets scored in visual class 4 with increasing doses of Quercetin in Quercetin and hyperthermia. Exposure to increasing doses of Quercetin revealed that the majority of comets were progressively distributed to the next visual category of higher DNA-damage. The average of tail moments in each category of cells was used as an indication of DNA damages. Figure 6 shows quantitative measurements of DNA damages by comet score program. Figure show DNA damage (DD) and the net induced DNA damage (DD-DD0) in the groups of Quercetin in combination with hyperthermia (43°C, 1 hour). As can be seen in both induced and net induced DNA damages of figure 6 DMSO diluent in combination with hyperthermia had no significant effect on DNA damages as compared to control (p>0.05). Furthermore, this figure shows that the induced DNA damages increased by increasing Quercetin concentration in combined treatment of DU145 cells with Quercetin and hyperthermia in spheroid cultures.

![Figure 4](image)

**Figure 4.** Effects of different concentrations of Quercetin and hyperthermia at 43°C for 1 hour on the colony forming ability of DU145 spheroid culture cells. Mean ± SEM of 3 experiments.
DISSCUSSION

Multicellular spheroid cells represent a three-dimensional model similar to cancerous cells. Many cell lines are capable of forming this structure. DU145 is an established cell line that is able to generate large, stable spheroids in liquid overlay culture (an easy and reliable technique to produce spheroids) [23]. Spheroids are used extensively for studying the effect of different treatments such as drugs, radiation, other factors and hyperthermia on tumor growth [24]. It has been reported that spheroids are more resistant than monolayer grown cells to hyperthermia which attributed to higher expression of HSPs [10]. Hyperthermia refers to the treatment of disease by raising the temperature of the body over the threshold temperature; the range of temperature is from 40°C to 48°C for a period of 1 hour or more. It has often used as an adjuvant with other established treatment modalities since it is able to sensitize cells to other treatments [25, 26]. Heat disturbs the function of transmembrane transport proteins and causes aggregation of misfolded proteins in cells matrix. It affects cytoskeletal organization, mitotic spindle, and other organelles and impedes the synthesis of DNA, RNA and protein [27, 28]. Although heat is not able to cause severe DNA damages, it is capable of blocking the repair of radiation-induced sub lethal cell damage, so enhances radiation – induced DNA fragmentation, by inhibition of DNA repair enzymes [29]. While synthesis of most cellular proteins is inhibited under heat conditions, heat shock proteins are synthesized. They bind to hydrophobic portion and avoid their interaction with other proteins which prevent their loss of function, lack of HSPs is correlated with cell death, and when they are inactivated increase of apoptosis is reported [30]. All these data recommend a close relation between HSP-synthesis and inhibition of hyperthermic cell death, especially apoptosis under stress conditions. Therefore hyperthermia kills cells by induction of protein denaturation and aggregation, and this event is antagonized by HSP expression which causes protein folding. DU145 cells in spheroid cultures show thermo-resistance at all levels of heat exposure which may be attributed to high expression of HSPs. So inhibition of HSP synthesis by applying HSP inhibitors may lead to thermo-sensitivity. Inhibition of HSP synthesis by applying Quercetin in spheroid cells followed with heat exposure will decrease their viability. Quercetin is a plant-derived flavonoid which is ubiquitous in our daily diet, especially in onion, tea, wine and apple [31]. Because of its health-promoting effects, Quercetin is recognized as a drug. It has been proved that Quercetin inhibits tumor growth and blocks the synthesis of HSP in cancerous cells [32]. In the present study, we have investigated the cytotoxic and genotoxic effects of different concentrations of Quercetin on spheroid cells exposed to heat (43°C, 1h). Quercetin is a water insoluble compound so in order to apply it in

**Figure 5.** Distribution of DNA migrations (stages 0 to 4) among DU145 cells of 100μm spheroids after treatment with quercetin and hyperthermia at 43°C for 1 hour. Data based on the analysis of 100 cells per slide, triplicate slides per samples.

**Figure 6.** Effects of different concentrations of Quercetin and hyperthermia at 43°C for 1 hour on induced DNA damages (DD) and net induced DNA damages (DD-DD0) of DU145 spheroid culture cells. Mean ± SEM of 3 experiments.
cell culture medium it was dissolved in DMSO. The final concentration of DMSO was 0.25% (v/v) due to its cytotoxic effects on cells. As shown in figure 1, we apply a range of different numbers of cells to obtain the most appropriate concentration of spheroid cells for colony counting. The number of colonies in each 60mm petri dish should be more than 50 colonies and less than 150 colonies to prevent colony overlapping; the optimum amount of plating cells per each 60mm petri dish was 3000 cells. It must be mentioned that the optimum number for monolayer culture is less than 3000 and around 500 cells per each 60mm petri dish. In figure 3 the viability of the cells was evaluated immediately after drug treatment and exposing to hyperthermia to make sure that reduction in cell survival was not due to sudden death of the cells. As shown in this figure, all cells are more than 95% viable. As illustrated in figure 4, the colony-forming ability of cells reduced by increasing Quercetin concentration in combination with hyperthermia in DU145 spheroid cell culture. Furthermore treating cells with DMSO has no significant effect on cell viability compared with the control. According to the graph, when we compare the number of colonies in cells treated with 500µM Quercetin with cells treated with fewer amounts, we observe a significant reduction in colony-forming ability. Figure 5 and 6 show the result of comet assay. In this graph tail moment is a parameter to measure the DNA damages. This figure represents increase in DNA damage and tail moment score by increasing the drug concentration in combined treatment of cells with hyperthermia. In this figure, we do not observe any significant differences between the control and the cells treated with DMSO. In the present research via administration of a range of different dosage of Quercetin, we have shown that Quercetin is able to inhibit HSP production in cells exposed to hyperthermia, and as a result, colonization reduced. We observed that Quercetin has cytotoxic and genotoxic effects on cells in a concentration-dependent manner, since it is an inhibitor of HSP production. Although heat is not able to cause severe DNA damages directly, but it is capable of inhibiting repair enzymes. HSPs prevent DNA damages by preventing the loss of function of DNA repair enzymes. When cells are exposed to heat, the expression of HSPs increased to cause cell survival. When these cells previously treated with Quercetin, the HSP production is inhibited so after heat exposure due to lack of these survival factors, cells are thermo-sensitive and cannot survive and we expected less viability, decrease in cologenicity and more tail moment (DNA damage). Our results presented here, emphasizes on the role of Quercetin as an inhibitor of HSPs. By applying higher concentrations of the drug, the levels of HSPs are reduced more and they showed more thermo-sensitivity.

CONCLUSION
Quercetin has been focused on as a reagent that could be combined with hyperthermia therapy. Our results showed that by using higher concentration of Quercetin in spheroid cultures, number of the colonies was reduced and tail moments were increased without any change in the viability of cells. Quercetin is a water insoluble material that cannot be dissolved in culture medium. For this reason, its transport across the cell membrane and transfer into the cells is very difficult. Hence, our suggestion for the next study is the use of copolymeric nanoparticles as carriers for the proper drug-dosage transport into cells.

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