Mild Hyperthermia (39°C) Attenuates Berberine Chloride Cytotoxicity against Osteosarcoma Cells
(Hipertermia Ringan (39°C) Keadaan Penerimaan Berberina Kesitotoksikan Klorida berbanding Sel Osteosarkoma)

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ABSTRACT

Many studies reported that antioxidants and dietary supplements increase tumour progress and reduce survival. Hyperthermia is an adjuvant treatment to sensitise cancer cells for radio- or chemotherapy. The current study was carried out to determine the effects of berberine chloride and hyperthermia on bone cancer cells. MG-63 osteosarcoma cells were treated with hyperthermia (39, 43 and 45°C, respectively) for 1 h. Then, the cells were treated with a low toxic dose of berberine chloride (80 µg/mL). After that, all treated groups were recovered at 37°C for 24 h. Finally, all groups were treated with hyperthermia (39, 43 and 45°C) for the second time (1 h) and were recovered for 3 h at 37°C. Cells exposed to hyperthermia without treatment of berberine chloride were used as hyperthermia control. Cells treated with 80 µg/mL of berberine at 37°C served as berberine control and cells incubated at 37°C were used as an untreated control. All treated groups showed significant apoptosis compared to the control group ($p<0.05$) except 39°C. On the other hand, mild hyperthermia treatment (39°C) resulted in a reduction of berberine-induced apoptosis ($p<0.001$). Severe and moderate hyperthermia did not show a significant increase in the rate of apoptosis compared to berberine treated cells. Mild hyperthermia treatment can effectively reduce berberine cytotoxicity and implying negative effects on cancer therapy.

Keywords: Apoptosis; berberine chloride; hyperthermia; osteosarcom

ABSTRAK

Banyak kajian melaporkan bahawa antioksidan dan makanan kesihatan meningkatkan kemajuan tumor dan mengurangkan survival. Hipertermia merupakan rawatan menormalkan untuk menyedarkan sel-sel kanser untuk radio - atau kemoterapi. Kajian ini telah dijalankan untuk menentukan kesan berberina klorida dan hipertermia ke atas sel kanser tulang. Sel osteosarkoma MG-63 dirawat dengan hipertermia (39, 43 dan 45°C, masing-masing) untuk 1 jam. Kemudian, sel dirawat dengan dos rendah toksik berberina klorida (80 µg/mL). Selepas itu, semua kumpulan yang dirawat telah pulih pada 37°C selama 24 jam. Akhirnya, semua kumpulan telah dirawat dengan hipertermia (39, 43 dan 45°C) kali kedua (1 jam) dan telah pulih untuk 3 jam pada 37°C. Sel yang terdedah kepada hipertermia tanpa rawatan berberina klorida telah digunakan sebagai kawalan hipertermia. Sel yang dirawat dengan 80 µg/mL berberina pada 37°C digunakan sebagai berberina kawalan dan sel yang dieram pada 37°C telah digunakan sebagai kawalan yang tidak dirawat. Semua kumpulan yang dirawat menunjukkan kesignifikanan apoptosis berbanding dengan kumpulan kawalan ($p<0.05$) kecuali pada 39°C. Selain itu, rawatan hipertermia ringan (39°C) telah mengurangkan apoptosis aruhan berberina ($p<0.001$). Hipertermia teruk dan sederhana tidak menunjukkan peningkatan yang ketara dalam kadar apoptosis berbanding dengan sel berberina yang dirawat. Rawatan hipertermia ringan berkesan boleh mengurangkan kesitotoksikan berberina, membayangkan kesan negatif terhadap terapi kanser.

Kata kunci: Apoptosis; berberina klorida; hipertermia; osteosarkoma

INTRODUCTION

Several studies have shown that exposure to hyperthermia results in increased apoptosis and reduced cellular viability in several mammalian cells (Aisha et al. 2012; Lv et al. 2013; Rong et al. 2000; Shellman et al. 2008; Trieb et al. 2007). Osteosarcoma, with an incidence of 0.03-0.2 per 100 000 per year, represents the most common primary malignant bone tumour, which arises predominantly from the metaphysis of the long bones of adolescents and young adults (Hogendoorn et al. 2010; López-Martínez et al. 2012).

Chemo- or radiotherapy cancer treatments are highly demanding on the patient's body. As such, some patients may use supplements like micronutrients (vitamins or polyphenols) to reduce the bystander effects of the therapy. According to Groeber (2009), approximately 60% of all cancer patients take supplements during their therapy without informing their oncologists. Sayin et al. (2014)

found that supplementing the diet with vitamin E and the antioxidant N-acetylcysteine (NAC) results in a marked increase in tumour progression and reduces survival in a mouse model of B-RAF- and K-RAS-induced lung cancer. Additionally, low doses of dietary antioxidants may enhance some cancer cells proliferation (ATBCCPSG 1994; Jha et al. 2012; Prasad et al. 2001).

Hyperthermia is used in combination with chemotherapy in several types of cancer including osteosarcomas, breast cancer, nasopharyngeal cancer, colorectal cancer, lung cancer, carcinoma of the oesophagus, bladder cancer and ovarian cancer (Gori et al. 2005; Hua et al. 2011; Kubota et al. 1984; Sugimachi et al. 1988; Tancredi et al. 2011; Verwaal et al. 2003; Yamamoto et al. 2012). This may be contributed by the enhancement of the oxygenation of otherwise hypoxic tumours, which led to increased susceptibility of the tumours to treatment (Hurwitz 2010; Matsuzaki et al. 2004). Furthermore, cells exposed to high-temperature will incur irreparable damage and initiate cell death (Hurwitz 2010).

Many studies are investigating the pharmacological effects of berberine. Berberine possesses anticancer, antimicrobial, antidiabetic, anti-oxidative, anti-inflammatory, and anticancer properties (Kuo et al. 2004; Park et al. 2015; Thirupurasundari et al. 2009; Wu et al. 2012; Yu et al. 2005). However, little is known about its effect on osteosarcoma cells, especially at low dose and in combination with hyperthermia. Therefore, the present study aimed to investigate the apoptotic effects of berberine chloride and hyperthermia separately and combined against human osteoblast-like-osteosarcoma cells MG-63. The regulation of heat shock proteins (HSPs) under hyperthermia and their possible involvement in attenuation of cytotoxicity of hyperthermia and berberine chloride were also investigated.

MATERIALS AND METHODS

Osteoblast-like-osteosarcoma cells MG-63 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Foetal bovine serum (FBS), phosphate buffered saline (PBS), DMEM/F12 medium, accutase, Trypan blue dye, and Penicillin-Streptomycin (pen-strep) were obtained from GIBCO-BRL Life Technologies (New York, USA). Berberine chloride was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). The RNeasy Mini kit and Sensiscripts RT kit were obtained from Qiagen (Hilden, Germany).

CELL CULTURE

Six groups of cells were treated with hyperthermia for 1 h: Two groups at mild, two at moderate and two at severe hyperthermia (39, 43 and 45°C, respectively). After finishing this session of hyperthermia, a low toxic dose of berberine chloride (80 µg/mL) was selected for treating one group of mild, moderate and severe hyperthermia (combination). After that, all treated groups

were recovered at 37°C for 24 h. Then, all groups were treated at hyperthermia (39, 43 and 45°C) for the second time (1 h) and were recovered for 3 h at 37°C. Cells were incubated at 37°C with medium and DMSO (< 1%) as a vehicle solvent as an untreated control. To show the effect of berberine chloride, another group was treated with 80 µg/mL of berberine and left for the same period at 37°C.

TRYPAN BLUE EXCLUSION ASSAY

Berberine chloride was dissolved in DMSO. Cells were seeded in t-25 flask at a density of $1 \times 10^4/cm^2$ and treated with several doses of berberine chloride (40, 80,120,160 and 200 µg/mL) for 24 h. In all groups, the final concentration of DMSO was less than 1%. The control group incubated with DMSO and medium. The cells were harvested, washed with cold PBS. For each treatment, the cells were counted on a haemocytometer with an equal volume of Trypan blue and serum-free medium. Each flask had two repeats of counting. The experiment was repeated three times.

QUANTIFICATION OF APOPTOSIS BY FLOW CYTOMETRY

The apoptosis rate of osteosarcoma MG-63 cells was quantified by flow cytometry after treating the cells in a t-25 flask. To harvest the cells, accutase was used with centrifugation, washed with cold PBS, stained with 1 µL annexin-V-FITC and 5 µL propidium iodide (PI) protected from light according to the manufacturer's protocol (BD Biosciences, San Jose, CA, USA) and then analysed with Becton FACSC flow cytometer (Becton Dickinson Corporation, USA). For each condition, 1×10^4 cells were studied in each experiment.

GENE EXPRESSION

The expression of mRNA for caspases 3, 8, 9, heat shock protein 27 and 70 was determined by RT^2 Profiler PCR Array system. For cDNA and genomic elimination, the RT^2 First Strand Kit (Qiagen, Germany) was used. A mixture containing 1 µg RNA was used for genomic elimination and cDNA synthesis as mentioned in the protocol. The prepared cDNA was added to the RT^2 SYBR Green qPCR Master Mix (Qiagen, Hilden, Germany). An amount of 25 uL of master mix was loaded onto the Custom RT^2 PCR Array plate for amplifying by using the iQ™5 Real-Time PCR detection system (Bio-Rad, USA). GAPDH and HPRT1 were used as reference genes. Data analysis was performed online using the student's t-test from three biological replicates (http://www.sabiosciences.com/pcrarraydataanalysis.php).

CASPASES ACTIVITY

MG-63 cells (5×10^3 cell/well), in DMEM/F12 and 10% FBS, and 1% pen-strep were plated in the white 96-well plate (SPL Life Sciences, Pocheon, Gyeonggi-do, Korea) and left overnight to allow the cells to attach. The media were changed with a fresh medium contains berberine chloride

80 ug/mL. DMSO was added as a vehicle control to the control group and groups treated with hyperthermia. The cells were treated as mentioned previously. Activity of caspases 3/7, 8, and 9 was estimated using luminescence based assay, Caspase-Glo 3/7 Assay, Caspase-Glo 8 Assay, and Caspase-Glo 9 from Promega (Madison, Wisconsin, USA). In short, after the treatment time, the reagent (100 uL) was added to white-well 96 well plates and incubated for 30 min at room temperature before performing luminescence reading. The luminometer was used Perkin-Elmer Victor X5 (Perkin Elmer, Waltham, MA, USA). Background reading was determined from wells containing medium alone and subtracted from all wells. The average result was calculated from three replicates.

ENZYME-LINKED IMMUNOSORBENT ASSAY

Heat shock proteins 90 alpha (Hsp90α) was obtained from eBioscience (San Diego, CA USA), heat shock protein 70 (Hsp70) from Abcam, (Cambridge, England, UK) and heat shock protein 27 (Hsp27) from Assaypro (UK) were measured by ELISA commercial kit according to the manufacturers' instructions. In brief, floating and attached cells were harvested, washed with ice cold PBS and lysed in 150 uL lysis buffer containing Halt Protease Inhibitor Cocktail (Thermo Scientific, USA). Protein extraction was stored in -80°C. Protein was quantified by NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA) and standardised concentration was used for all the samples. The obtained absorbance for samples blanks and standards were measured by a plate reader Perkin-Elmer Victor X5 multilabel reader (Perkin Elmer, Waltham, MA, USA). The samples optical density (OD) was compared to a standard curve using an online website to get the protein concentration (http://elisaanalysis.com/app).

STATISTICAL ANALYSIS

SPSS V.22 statistical software and Excel 2010 were used for the statistical analysis and graphical assessments. Statistical tests were performed using the student's t-test and one-way ANOVA with Tukey's posthoc tests after running a normality test. The results are presented as means ± SD, and all measurements and analyses were carried out in triplicate.

RESULTS

THE CYTOTOXICITY OF BERBERINE ON MG-63 HUMAN OSTEOSARCOMA CELLS

Figure 1 shows the cell viability of MG-63 human osteosarcoma cells that was measured after treatment with several doses of berberine chloride (0-200 μg/mL) for 24 h. The response of MG-63 cells to berberine chloride was dose-dependent. 80 μg/mL was chosen as a low toxic dose (viability 86.8%) to investigate the response of MG-63 human osteosarcoma cells to berberine chloride alone and in combination with mild, moderate and severe hyperthermia.

BERBERINE, MODERATE AND SEVERE HYPERTHERMIA-INDUCED APOPTOSIS

Figure 2 shows that the cell viability of the berberine group (37°C+berberine) dropped to 83.7%. The measured cell viability of MG-63 cells after exposure to mild, moderate, and severe hyperthermia (39, 43, and 45°C) was 93.5, 89.3, and 83.6%, respectively, while the combined treatment of hyperthermia with berberine caused a significant reduction of cell viability to 89.5, 82, and 85.9%, respectively when compared to the 37°C control of 94.1%. Comparing 45°C alone with 45°C combined with berberine, the viability increased slightly to 85.9%.

Comparing cell viability between berberine treatment alone at 37°C and combination groups shows that in berberine groups, viability was 83.7%, and no significant difference was observed, except that mild hyperthermia combined with berberine showed a significant increase compared to the berberine group (89.5%, $p<0.001$). This shows that mild hyperthermia attenuated the cytotoxicity of berberine. Figure 2 also shows that only moderated hyperthermia reduced cell viability from 89.3% to 82% after combining with berberine ($p<0.001$) but no significant increase in the rate of apoptosis.
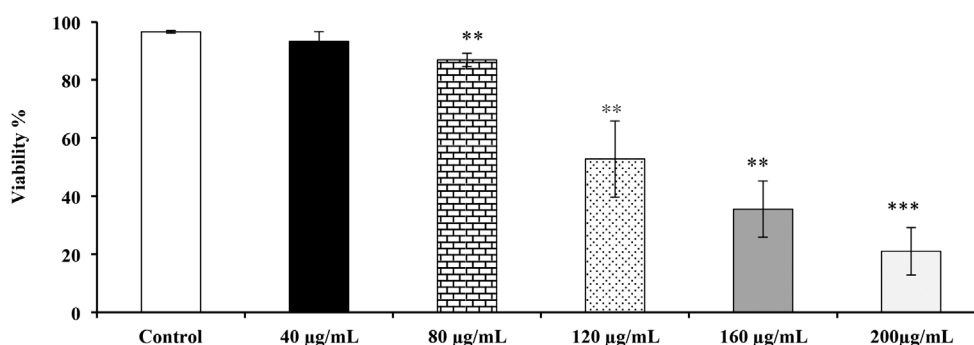

FIGURE 1. Effect of berberine chloride on the cell viability of MG63 cells. Cell viability after berberine incubation for 24 h was determined using trypan blue staining. Viability was expressed as mean percent of viable cells ±SD of three biological replicates. Statistical analysis by Student's t-test showed significant *$p<0.05$, * * $p<0.01$ and ***$p<0.001$ versus unstimulated control group
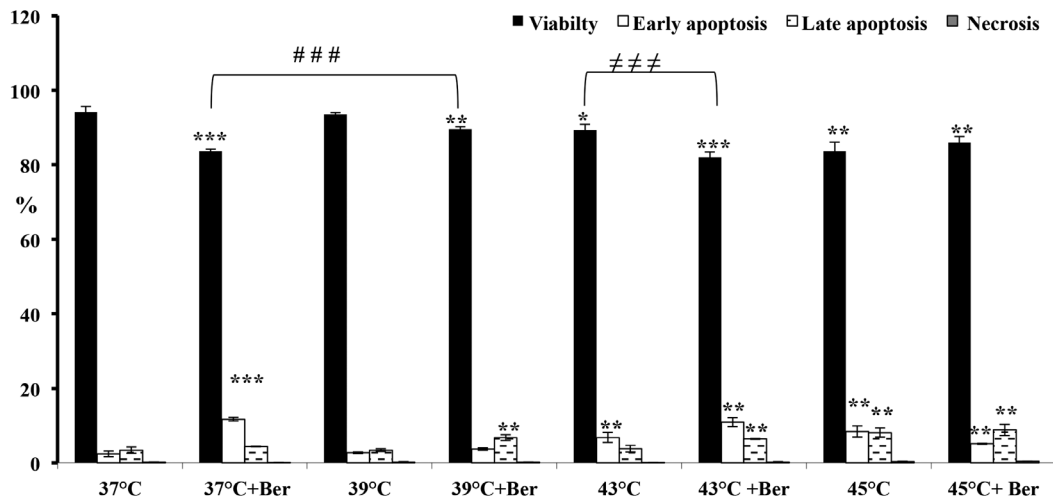
FIGURE 2. Berberine and hyperthermia induced apoptosis of MG-63 cells. Data represents the mean percentage of viable, early and late apoptotic cells ± SD (*n*=3). Using Student's t-test the significance was set as *p<0.05, * * p<0.01, ***p<0.001 when comparing treatment with 37°C (control). #p< 0.001. vice 37°C+ berberine using One-way ANOVA with Tukey's posthoc tests. ≠p< 0.001 (Moderate hyperthermia vice moderate hyperthermia+ berberine)

HYPERTHERMIA AND BERBERINE SUPPRESSED CASPASE EXPRESSION AND ACTIVATION

Figure 3(A) shows the biological effect of hyperthermia and berberine chloride on effectors caspases. Both treatments, separate and in combination, did not result in a significant increase in caspase 3/7 activity compared with untreated control. Caspase 8 was downregulated in all groups while caspase 9 showed significant down-regulation in all groups, except those treated with 39 and 45°C were down-regulated, but the differences were not significant. The mRNA for caspases 3, 8, 9 were down-regulated (Figure 3(B)).

MODERATE AND SEVERE HYPERTHERMIA INDUCED Hsp 27 AND Hsp70 EXPRESSION

Berberine alone incubated at body temperature (37°C) did not affect the cellular protein expression of Hsp90α and 70 while it downregulated the expression of Hsp27. Mild hyperthermia showed the same pattern: No effect on the expression of Hsp90α and 70 and downregulation of Hsp27. Moderate and severe hyperthermia applied alone, significantly upregulated the expression of Hsp27 and 70 in osteosarcoma cells while severe hyperthermia additionally downregulated the expression of Hsp90α. When combining moderate and severe hyperthermia with berberine, the same pattern of expression of the heat shock proteins was observed: Increased expression of Hsp70 and Hsp27 when compared with control. Moderate hyperthermia in combination with berberine showed downregulation of Hsp90α expression compared with control and berberine alone. Severe hyperthermia with berberine further reduced the expression of Hsp90α when compared with severe hyperthermia alone. In the moderate hyperthermia plus berberine group, a reduction of Hsp70 and 27 was observed when compared with moderate hyperthermia alone. However, the protein

levels did not reach the level of the untreated control cells (Figure 4(A)).

The gene expression results showed that Hsp70 was decreased in 37°C combined with berberine, mild hypothermia combined with berberine, whereas severe hyperthermia showed a two-fold increase with significant value when applied alone (p<0.01) as shown in Figure 4(B). Interestingly, we observed that groups treated with berberine in combination with hyperthermia caused more reduction in gene expression of Hsp70. On the other hand, Hsp27 mRNA decreased in all groups except that moderate hyperthermia (43°C) showed a slight increment.

DISCUSSION

Apoptosis is a process that regulates cell turnover. It is also known as programmed cell death. Apoptosis takes place in both pathological and physiological conditions. It plays a vital biological role in processes such as tissue homeostasis, embryonic development and cell proliferation (Elmore 2007). The current study found that moderate and severe hyperthermia resulted in the induction of apoptosis in osteosarcoma cells separately and in combination with berberine chloride. These results are consistent with a previous study on osteosarcoma cells involving MG-63 (Trieb et al. 2007). The combination of mild hyperthermia with berberine decreased the berberine toxicity significantly compared to cells treated with berberine alone, whereas severe hyperthermia combination led to a slight decrease of berberine toxicity. On the other hand, moderate hyperthermia enhanced berberine toxicity slightly.

When investigating drug-drug interactions, or in this case antioxidant-treatment interactions, it is not always clear what effects what. Zaidi et al. (2004) found that whole-body mild hyperthermia (39°C, 1 h) impaired radiation-induced apoptosis in mice. Bagewadikar et
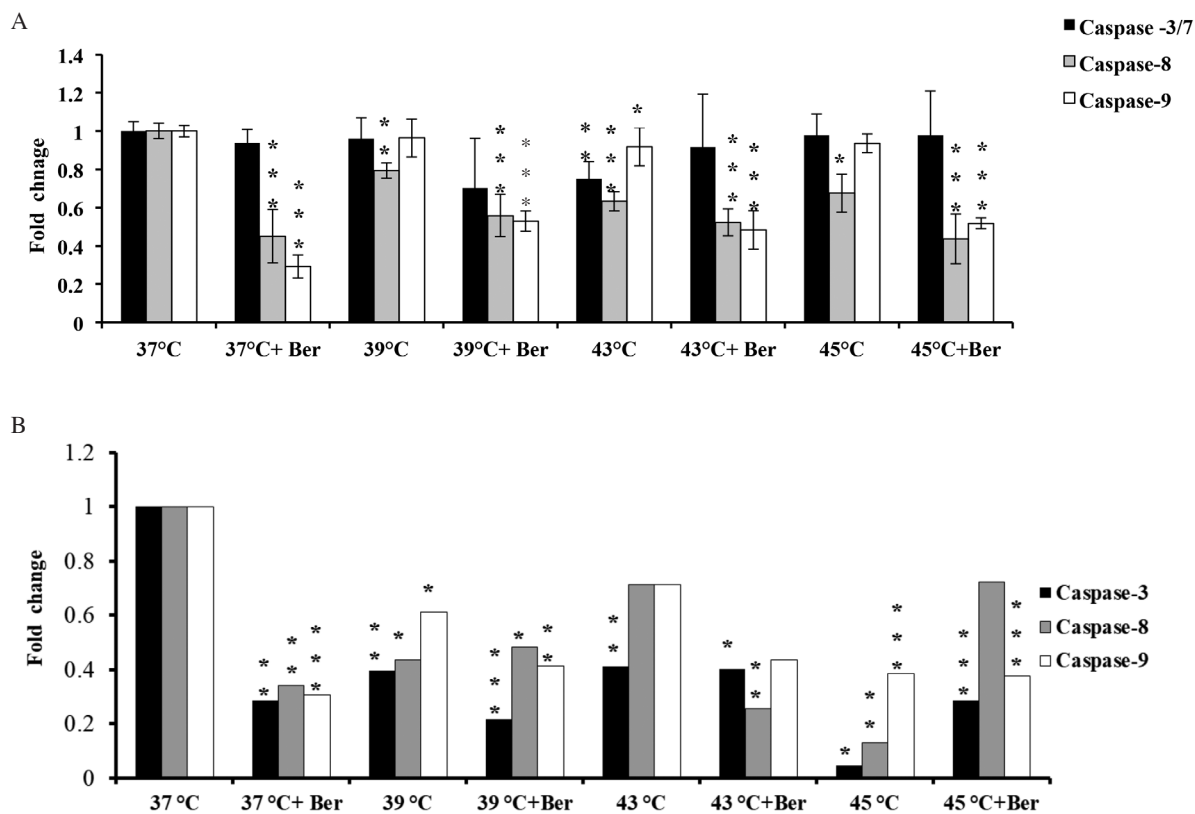
FIGURE 3. (A) Hyperthermia and berberine chloride supressed activity of caspases 3/7, 8 and 9. (B) Hyperthermia and berberine chloride supressed mRNA of caspase 3,8 and 9. PCR array was used to measure mRNA levels. Data are expressed as means (*n=3*). *p<0.05, * * p<0.01 and *** p<0.001 versus unstimulated control groups. Student's t-test was used
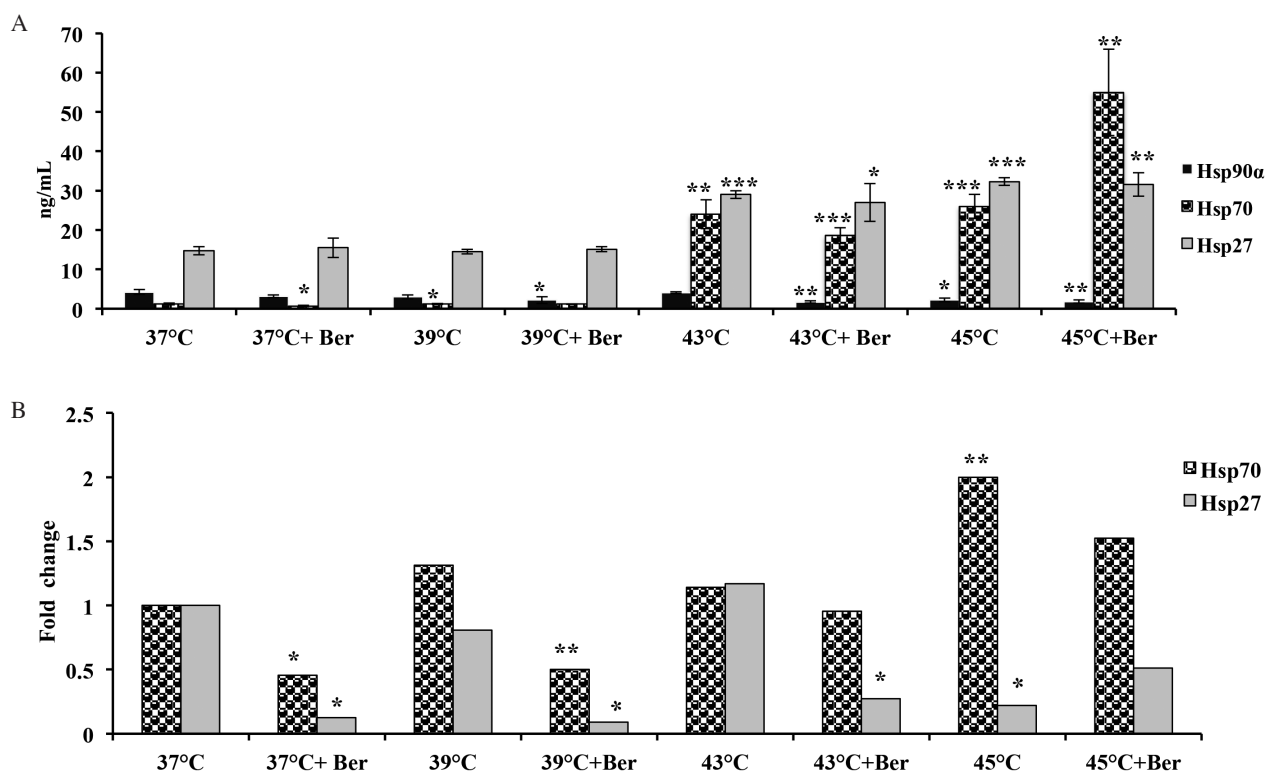

FIGURE 4. (A) Moderate and severe hyperthermia induced Hsp70 and Hsp27 expression (B.) Severe hyperthermia induced Hsp70 mRNA expression. The analysis was performed by RT2 Profiler PCR Array System. Data represents the mean fold change (*n*=3). Statistical analysis by Student's t-test showed significant *p<0.05, * * p<0.01 and ***p<0.001 versus unstimulated control groups

al. (2001) reported that prior whole-body hyperthermia treatment could effectively reduce N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) cytotoxicity in mice. Our results are consistent with these studies. Hence, mild hyperthermia (39°C) reduced the apoptotic rate of MG-63 cells exposed to berberine by around 6%. Upon combining moderate and severe hyperthermia with berberine, the rate of cell viability showed no significant difference compared with the group treated with berberine alone. Furthermore, moderate hyperthermia sensitised the cells for berberine chloride, but the differences in cell viability were not significant compared with the group treated with berberine alone.

Caspases are specific cysteine-dependent aspartate-directed proteases, or cysteine-aspartic proteases are a group of 12 different cysteine proteases. They can be subdivided into initiator, executioner and inflammatory caspases which play essential roles in cell death and inflammation (McIlwain et al. 2013). Initiator caspases (8 and 9) activate executioner caspases (caspase-3 and 7) that coordinate their activities to demolish critical structural proteins and to activate other enzymes, leading to apoptosis (Degterev et al. 2003). On the other hand, increased levels of HSPs have been found in tumour cells where they are involved in various mechanisms including carcinogenesis, cell proliferation and survival.

It has been reported that overexpression of Hsp70 inhibits many pathways of cell death (Murphy 2013). Hsp27 plays many physiological roles such as suppression of apoptosis and defence against oxidative stress (Mymrikov et al. 2011). Additionally, Hsp90 represents an attractive target for cancer treatment because of its role in stabilising many client oncoproteins (Gallerne et al. 2013). In the present study, at the protein level, Hsp70 and Hsp27 were significantly upregulated in moderate and severe hyperthermia and their combined groups, whereas Hsp90α was downregulated in all groups except in moderate hyperthermia.

In summary, this significant increase of HSPs expression might explain the absence of the synergetic or additive apoptotic effect when berberine was combined with moderate and server hyperthermia. Caspase 3, 8, and 9 were suppressed in all groups at transcription levels. The activity of caspases 3/7 showed a slight reduction in mild and moderate hyperthermia. Taken together, these results may indicate caspase-independent apoptosis. Wang et al. (2012) reported that berberine induces caspase-independent cell death in colon tumour cells. The results of our study are consistent with this study. Some studies reported that ROS generation mediates berberine-induced apoptosis in human cancer cells (Chen et al. 2009; Meeran et al. 2008; Xie et al. 2015). Another study reported that berberine was able to induce apoptosis and DNA damage of MG-63 cells (Zhu et al. 2014). According to this finding, the significant induction of heat shock proteins under hyperthermia suggests a protective mechanism by which HSPs repair DNA damage (Sottile & Nadin 2017). Based on these studies, the attenuated toxicity of berberine or

absence of a significant synergistic effect in the present study could be attributed to the significant induction of HSPs proteins by mild and severe hyperthermia.

CONCLUSION

These findings raise concerns for clinical use of mild hyperthermia. Furthermore, low doses of berberine chloride have no significant additive toxic effect in combination with moderate and severe hyperthermia, and therefore, may be safe at low doses to be taken together with hyperthermia therapy to reduce the bystander effect of the treatment. Further studies are recommended to determine the protective mechanism of mild hyperthermia against berberine chloride.

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