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Berberine Chloride and Hyperthermia Promote Osterix Expression and Suppress Cell Cycle Genes in Osteosarcoma Cells

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Objective: To investigate the effects of berberine chloride and hyperthermia on the expression of Osterix (Osx), Runt-related transcription factor 2 (RUNX-2), receptor activator of nuclear factor kappa-B ligand (RANKL), cyclin-dependent kinase 2 (CDK2), CDK4, interleukin (IL)-6 and IL-11 genes.

Methods: Osteosarcoma cells (MG-63 cells) were treated with three different hyperthermia conditions for 1 h: two groups with mild, two with moderate and two with severe hyperthermia (at 39, 43 and 45 °C, respectively). Berberine chloride (80 µg/ml) was used for treating one group of mild, moderate and severe hyperthermia (combination). All treated groups were recovered at 37°C for 24 h. Another exposure to hyperthermia (1 h) and recovery at 37°C for 3 h were applied. Gene expression levels were then determined using RT² PCR profiler arrays.

Results: The expression of Osx was highly upregulated in all groups except in the groups treated with severe hyperthermia and mild hyperthermia in combination with berberine chloride. Mild hyperthermia alone induced a slight increase in the expression of RUNX2, whereas severe hyperthermia significantly suppressed RUNX2 levels. Berberine alone significantly induces the up-regulation of RANKL expression. On the other hand, CDK2 mRNA was downregulated in all groups. CDK4 was equally regulated in mild hyperthermia compared to the control group. However, the expression was significantly downregulated in other groups, especially in severe hyperthermia. IL-6 was significantly upregulated in berberine group and all groups of combinations, whereas mild and moderate hyperthermia significantly induced the expression of IL-11 mRNA.

Conclusions: Hyperthermia and berberine chloride can promote osteosarcoma cells differentiation and suppresses cell cycle genes.

Keywords: Berberine chloride, Hyperthermia, Osterix, RUNX2, Cell cycle, Osteosarcoma

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1. Introduction

Osteoblasts and osteosarcoma cells originate from mesenchymal stem cells. Although osteoblasts undergo multiple differentiation steps, the differentiation of osteosarcoma cells is impaired so that they remain as immature osteoblast-like cells (1). Osterix (Osx) and Runt-related transcription factor 2 (RUNX2) are osteoblast-specific transcription factors necessary for the differentiation of osteoblasts and bone formation. Stein et al. (2) concluded that RUNX2 has a dual function in osteoblast proliferation and differentiation. It enhances their maturation by supporting the exit from the cell cycle and attenuation of osteoblast growth and activates the genes of osteoblast maturation to promote bone cell phenotype development and lineage commitment (2). RUNX2 induces BAX expression and increases the sensitivity of sarcoma osteogenic (Saos2) cell lines to apoptosis (3). Inversely, Martin et al. (4) found overexpression of RUNX2 mRNA in patients with osteosarcoma, leading to a poor response to chemotherapy compared to the good response in patients with other types of tumors. Osx is an important osteoblast-specific transcription factor that plays an essential role in osteoblast proliferation and differentiation (1, 5). It binds to substance P (Sp)-binding sites on gene promoters to regulate their expression. According to Cao et al. (6), Osx transcript decreased in three human and two mouse osteosarcoma cell lines compared to normal cell lines. Moreover, transfection of a mouse osteosarcoma cell line with the Osx gene resulted in the inhibition of tumor incidence, growth and volume (6).

Osteoclasts are cells derived from the monocyte/macrophage lineage and play an important role in bone metabolism through their engagement in the bone resorption process. The tumor necrosis factor (TNF) family consists of several ligand-receptor proteins that play a role in several cellular processes including apopto-

sis, homeostasis and development (7). One of such proteins is the receptor activator of nuclear factor kappa-B ligand (RANKL), which is very important for the activation, formation, and function of osteoclasts (8). RANKL is expressed and synthesized by different types of cells such as osteoblasts and bone marrow stromal cells (8). On the other hand, RANK, the receptor for RANKL, is another member of the TNF family, which is mainly expressed on the cells of the monocyte/macrophage lineage such as osteoclasts and pre-osteoclasts (7). The RANKL/ RANK interaction is essential for osteoclastogenesis as it stimulates the differentiation and maturation of osteoclasts (9). RANK expression is regulated by TNF- α , parathyroid hormone-related protein, transforming growth factor- α , interleukins (IL-1, IL-6 and IL-8) (10). Activated osteoclasts attach to the bone and release various proteases, which facilitate the resorption of the non-mineralized constituents of bone (11).

Berberine is a quaternary ammonium salt that belongs to the family of isoquinoline alkaloids. It is found in several plant species such as *Berberis aristata*, *Hydrastis canadensis* (golden-seal) and *Coptis chinensis* (12). Several studies have shown the anticancer properties of berberine against different types of cancers, including leukemia, osteosarcoma and colon cancer (13–15). On the other hand, hyperthermia (39–45°C) has been shown to be a powerful enhancer for chemo- and radiotherapies through the elimination of treatment-resistant tumor cells (16).

Mantena et al. (17) reported that treatment of human prostate cancer cells with berberine led to G1-phase arrest with suppression of cyclin-dependent kinase (CDK) 2, CDK4, and CDK6 proteins. Additionally, a recent study showed that berberine alone or in combination with doxorubicin led to G2/M arrest in human breast cancer cells (18). However, published data on the effects of berberine chloride, hyperthermia or their combination on osteogenesis and tumor cell differentiation are still very scarce. There-



fore, the present study aimed to investigate the effects of berberine chloride and various heat conditions on the gene expression of *Osx*, *RUNX-2*, *RANKL*, cyclin-dependent kinase 2 (*CDK2*), *CDK4*, *IL-6* and *IL-11*.

2. Methods

2.1. Cell culture

Human MG-63 Osteoblast-like osteosarcoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F-12) with 10% fetal bovine serum and 1% streptomycin and penicillin (Life Technologies, Grand Island, NY, USA). Culture media were changed every 48 h. The cells were sub-cultured in an atmosphere of 5% CO₂ at 37°C overnight and then transferred to a different incubator with the required pre-adjusted temperature.

2.2. Hyperthermia treatment and berberine chloride

Six groups of MG-63 cells were treated with hyperthermia for 1 h: two with mild, two with moderate and two with severe hyperthermia (at 39, 43 and 45°C, respectively). After treating the cells with hyperthermia, a low dose of berberine chloride (80 µg/ml) was used for treating one group of mild, moderate and severe hyperthermia (combination). Then, all treated groups were recovered at 37°C for 24 h, and all groups were treated with hyperthermia (at 39, 43 and 45°C) for a second time (1 h) and were recovered at 37°C for 3 h.

2.3. PCR gene expression arrays (RT² PCR profiler arrays)

The RNeasy Mini Kit with on-column DNase treatment step (Qiagen, Hilden, Germany) was used for RNA extraction. Genomic DNA was eliminated by mixing 2 µl of DNA elimination

buffer (Qiagen, Germany) with 1-8 µl of total RNA. The mixture was incubated at 42°C for 10 min. For cDNA synthesis, the RT² First Strand Kit (Qiagen, Hilden, Germany) was used. Each reaction contained 1 µg of DNA-free RNA (10 µl), 1 µl primers solution and external control mix, 4 µl 5x RT buffer, 3 µl dH₂O and 2 µl RT enzyme mix (Qiagen, Hilden, Germany). This mixture was incubated in a Mastercycler Gradient machine (Eppendorf, Hamburg, Germany) at 42°C for 15 min. and the reaction was terminated by increasing the temperature to 95°C for 5 min. Prior to running the PCR array, 91 µl of distilled H₂O was added to the cDNA and mixed gently.

A microliter of the cDNA product was loaded into each well of the RT² profiler PCR array after mixing with RT² PCR Master Mix (25 µl) (Qiagen, Hilden, Germany) and amplified using the iQ[™] 5 Real-Time PCR Detection System (Bio-Rad, USA). Three biological replicates were investigated for each group. The expression levels of *Osx*, *RUNX-2*, *CDK2*, *CDK4*, *IL-6*, *IL-11* and *RANKL* were determined. Gene expression was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*; catalog number: PPH00150) and hypoxanthine phosphoribosyltransferase 1 gene (*HPRT1*; catalog number: PPH01018) as reference genes using the RT² profiler PCR arrays.

2.4. Statistical analysis

Data were analyzed using Student's *t*-test to compare every treated group to control by using a web-based SABioscience RT² Profiler PCR Array Data Analysis (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>). Differences were considered statistically significant at *P* values < 0.05.

3. Results

Figure (1) shows the effect of hyperthermia and berberine chloride on the expression of *RUNX2*, *RANKL* and *Osx* genes. Mild hyperthermia in-



duced a slight increase in the expression of RUNX2 mRNA, whereas severe hyperthermia significantly suppressed the levels of expression. Moderate hyperthermia alone and mild hyperthermia alone or in combination with berberine increased gene expression compared to the control. Additionally, berberine chloride alone was more effective in increasing RANKL

expression. Furthermore, the combination of berberine with mild hyperthermia attenuated the inducing effect of berberine chloride. Expression of Osx was highly induced in all groups, except for the combination groups of severe hyperthermia and mild hyperthermia plus berberine chloride.

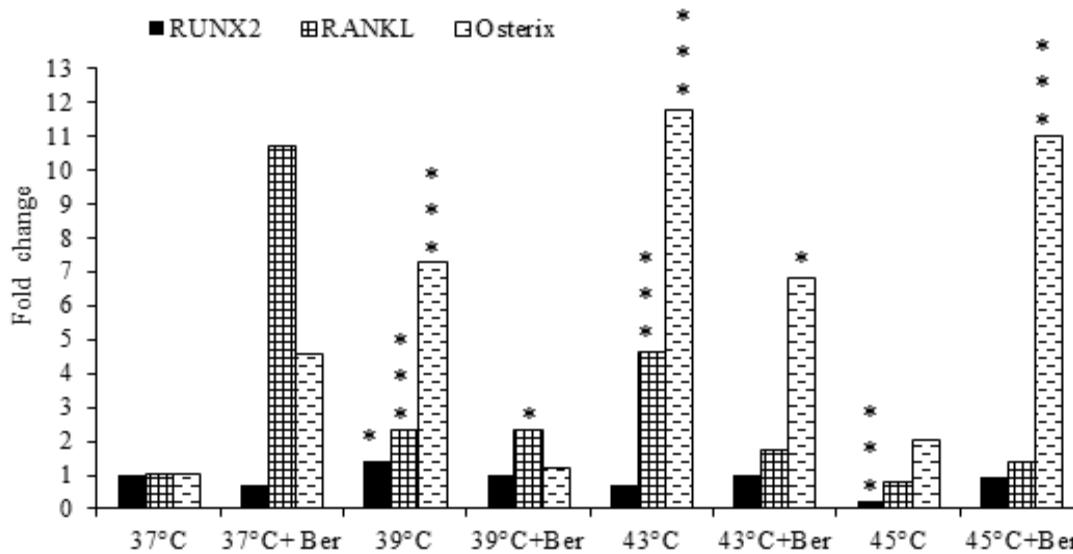


Figure 1. Effect of hyperthermia, berberine chloride and their combination on gene expression of RUNX2, RANKL and Osx

Figure (2) shows the long-term effect of berberine chloride, hyperthermia and their combination on the expression of cell cycle genes. CDK2 mRNA was downregulated in all groups. CDK4 mRNA was equally regulated in

mild hyperthermia compared to the control group. However, the expression was downregulated in other groups, especially in the severe hyperthermia group ($P < 0.5$).

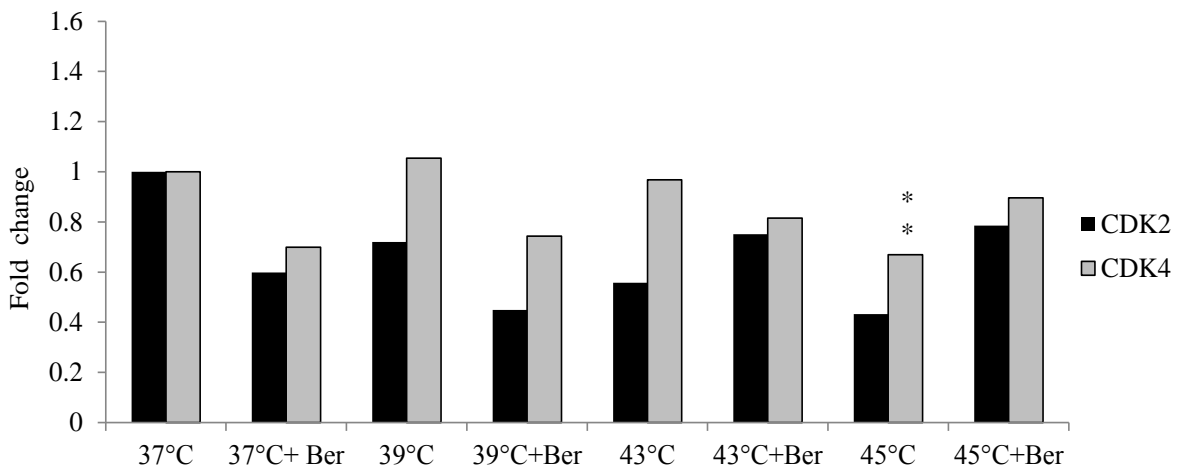


Figure 2. Effect of hyperthermia, berberine chloride and their combination on gene expression of CDK2 and CDK4



IL-6 was highly upregulated, with statistically significant differences in the berberine chloride group and all combination groups (hyperthermia + berberine chloride). Mild and moderate hyperthermia significantly induced the expression of IL-11 mRNA. However, such induc-

tion was attenuated after combination with berberine chloride, which resulted in a significant downregulation with mild hyperthermia and returning to the basic level with moderate hyperthermia (Figure 3).

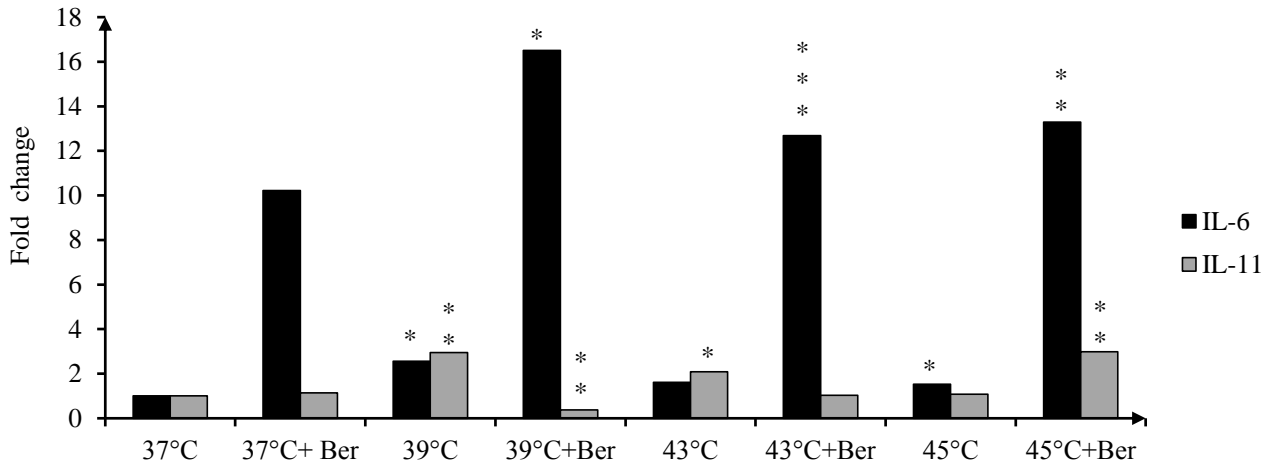


Figure 3. Effects of hyperthermia, berberine chloride and their combination on gene expression of IL-6 and IL-11

4. Discussion

In the present study, berberine chloride led to the suppression of CDK2 and CDK4 mRNA expression. This finding is in agreement with a previous study that showed G1-phase arrest with suppression of CDK2, CDK4 and CDK6 proteins in human prostate cancer cells exposed to berberine chloride (17). Additionally, a recent study showed that berberine alone or in combination with doxorubicin led to G2/M arrest in human breast cancer cells (18).

In the present study, severe hyperthermia alone significantly suppressed RUNX2 expression, which declined to near normal levels of control after combination with berberine. It is worth mentioning that thermal stress induces the expression of alkaline phosphatase, RUNX2 and *Osx* in mouse osteoblast MC3T3-E1 cells (19). *Osx* was upregulated in all groups. Overall, the present study showed that hyperthermia may induce differentiation of osteosarcoma

cells. Kajiya et al. (19) reported that photo-thermal stress promotes mineral deposition in enhanced tooth-extracted sockets. On the other hand, it has been reported that berberine activates RUNX2 by p38 mitogen-activated protein kinase, leading to increased expression of osteogenic marker genes involving osteocalcin and osteopontin (20). In the present study, berberine-induced upregulated levels of *Osx* are consistent with the findings by Lee et al. (20) in normal osteoblasts.

RANKL is important for osteoclastogenesis (21), where both RANKL and osteoclastogenesis were found to be downregulated by berberine (22). Therefore, berberine has been suggested to be useful in the prevention of osteoporosis (22). It is noteworthy that increased osteoclastic activity in osteosarcoma is a sign of increased tumor cell invasion (11). This could explain the inhibitory effect of berberine on osteoclast formation in osteosarcoma and, therefore, its protective action against cancer cell invasion.



In the present study, severe hyperthermia suppressed RANKL expression, whereas berberine, mild hyperthermia and the combination of mild hyperthermia with berberine and moderate hyperthermia upregulated RANKL expression by more than twofold. This is in contrast to a finding by Zhou et al. (22), where berberine in normal osteoblasts reduced RANKL expression. Xue et al. (23) also showed suppression of osteoclast differentiation in a co-culture model system. Suppression of RANKL has been suggested as a good strategy for the treatment of aggressive osteosarcomas (24). Although berberine increased RANKL expression in osteosarcoma cells when given alone, it did not attenuate the effect of severe hyperthermia on RANKL expression, and therefore, had probably no negative effect on the treatment of osteosarcoma with hyperthermia. However, this requires further in-depth molecular studies because berberine significantly increased the expression of IL-6 and IL11, regardless of being given alone or in combination with hyperthermia. It is noteworthy that IL-6 and IL-11 are strongly involved in the upregulation of RANKL, and consequently, in osteoclastogenesis (11).

This study was conducted on one cell line with a single dose of berberine chloride, and no protein expression assays were performed due to the time and budget shortages. Thus, further studies are recommended to investigate the response of more osteosarcoma cell line types to the combined effects of berberine chloride and hyperthermia.

5. Conclusions

Berberine chloride suppresses cell cycle genes when used alone and in combination with mild or moderate hyperthermia. In addition, it induces *Osx* expression when used alone or in combination. Therefore, berberine chloride and hyper-

thermia could be effectively used in promoting osteosarcoma differentiation and, therefore, help in the treatment of osteosarcoma.

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Authors' contributions

MN, AA and GF contributed to the study design and data analysis. MN performed the laboratory experiments. All authors contributed to drafting, revising and approval of the final submitted manuscript.

Competing interests

The authors declare that they have no competing interests associated with this article.

Ethical approval

Not required.

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