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RESEARCH ARTICLE

Human MG-63 osteosarcoma cells responses to long and short term hyper- and hypothermia stress

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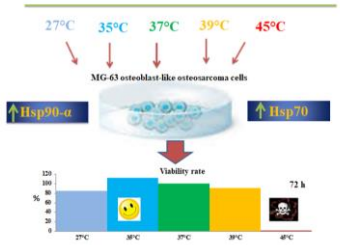
Graphical abstract



Abstract

Hyper- and hypothermia are utilized as treatment modalities in cancer treatment or as a protection against ischemia-reperfusion induced cell damage. The under-lying mechanism of hyper- and hypothermia, on cell death in osteosarcoma cells are not well understood. The aim of this study is to investigate the short- and long-term effects of various severities of hyper- and hypothermia on osteoblast-like osteosarcoma cells (MG-63). MG-63 cells were treated with mild and severe hyper- and hypothermia for short, medium and long-term periods. Severe hypothermia and hyperthermia showed a time-dependent toxicity; hence viability was reduced in a significant manner at all time points and the cells were undergoing apoptosis. Mild hypothermia, on the other hand, showed a protective effect and long term exposure increased the cell viability. Severe hyperthermia induced significant DNA damage at all time points. Caspase 3/7 activity showed a significant increase at 1 h of severe hyperthermia and 72 h of severe hypothermia ($p<0.05$). Hsp90 expression was significantly increased at 72 h of mild hyperthermia ($p<0.01$), whereas Hsp70 showed a significant increase after 24 and 72 h ($p<0.01$ and $p<0.001$). Hsp27 mRNA was increased significantly at 24 h only under mild hyperthermia ($p<0.01$). In conclusion, hyperthermia especially severe hyperthermia induced cellular stress in MG-63 cells leading to apoptosis. Hypothermia, on the other hand caused severe cell stress only when the cells were challenged for a prolonged period with severe low temperatures.

Keywords: hyperthermia, hypothermia, osteosarcoma, heat shock proteins, cold shock proteins

INTRODUCTION

Hyper- and hypothermia are extreme temperatures which can occur physiologically when the body's thermoregulation fails due to e.g. extreme surrounding temperatures in the summer or winter. Hyper- and hypothermia, however, are also treatment modalities. Hypothermia for example is been used to protect the heart and the brain during open cardiac surgery while hyperthermia on the other hand is used as a sensitizer for chemotherapeutic drugs by oxygenating otherwise hypoxic tumor tissue which then leads to increased susceptibility of the tumor to treatment [1,2].

Normal, healthy cells respond to extreme temperatures with a well-orchestrated molecular response involving the up-regulation of heat and cold shock proteins. While the mechanism and function of heat shock proteins are well established, the function and regulation of the cold shock proteins are less well understood. Both, heat and cold shock proteins are molecular chaperones that play an important role in the maintenance of correctly folded proteins. As cancer cells are under continuous stress, certain heat shock proteins are up-regulated to maintain cell viability, drive cancer cell differentiation & cell proliferation, prevent cell death and facilitate metastasis [3]. Zeng et al. (2009) found that the down-regulation of cold shock proteins such as cold inducible RNA binding protein (CIRBP) and RNA binding motif protein 3 (RBM3) with or without temperature treatment increases cell death and susceptibility to cancer drugs in prostate cancer cells [4].

Environmentally caused cell stress for example in form of hypo- and hyperthermia can burden the cells metabolism so much, that the cell death pathway is initiated. As cancer cells are already under a high level of stress, they should be more susceptible to temperature than normal cells, however some cancer cells like A549 (human lung adenocarcinoma) are thermo-tolerant [5]. The reason for different responses to temperature is not clear.

Osteosarcoma, a relatively uncommon malignancy, is a mesenchymal tumor of the bone with an incidence of 0.03–0.2 per 100,000 per year [6]. With this, it is the most common primary malignant bone tumor, which mainly arises from the metaphysis of the long bones of adolescents and young adults [6,7]. Osteosarcoma has a high resistance to chemo- and radiotherapy. Furthermore, it has been demonstrated that osteosarcomas are resistant to anoikis [8], a form of cell death that occurs when the cell looses its adhesion and contact with neighboring cells or the extracellular matrix for example during hyperthermia treatment.

Trieb et al. (2007) reported a significant reduction in cell growth of osteosarcoma cells after a heat shock treatment at 42°C [9]. Rong and Mack (2000) reported that hyperthermia induced apoptotic changes in Dunn osteosarcoma cells [10]. Furthermore, Kanamori et al. (2003)

found that hyperthermia showed a reduction of DNA synthesis in murine Dunn osteosarcoma cells [11]. Despite the above mentioned positive effects of hyperthermia on osteosarcoma cells, little is known about the underlying molecular mechanism leading to cell death or the development of resistance.

Hypothermia has mainly been proven to be protective in certain medical situations such as brain or open heart surgery, where it leads to a reduction of oxygen consumption and basal metabolic rate and so preventing damage to the brain [12]. So far very few studies using hypothermia on osteosarcoma cells can be found. It is well established that cell growth of normal mammalian cells is inhibited due to a cell cycle arrest at the G1 phase and apoptosis when cells are exposed to temperature below 37°C [13]. The cells enter a stage of quiescence first, where proliferation is inhibited, but the cell remains metabolically active (35°C). Temperatures below 25 °C induce apoptosis [13]. Furthermore, some studies found that hypothermia lead to a large decrease in heat shock proteins Hsp110, Hsp70, Hsp105, IL-8 and TNF-alpha, over-expression of cold inducible RNA binding protein (CIRBP) and up-regulation of apoptosis-specific protein (ASP) [14-16]. Few studies were published by our institute about the effect of hypo and hyperthermia on the normal osteoblast cell line [17-19]. Thus, the current study was conducted on osteosarcoma cells alone.

Overall there is a lack of data about effect of hypo- and hyperthermia on bone cancer cells, so this study was performed to investigate the different mechanisms. In this study the effect of long, moderate and short term exposure of osteoblast–like osteosarcoma MG-63 cells to mild, and severe hyper- and hypothermia were investigated. The toxicity effects, induction of apoptosis, gene and protein expresstion and DNA damage response were determined.

EXPERIMENTAL

Cell culture

Osteoblast-like osteosarcoma cells MG-63 were obtained from the American Type Culture Collection (ATCC, Manassas, USA) and were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin (Life Technologies (Grand Island, USA). The media was changed every 48-72 h. For the investigation of MG-63 cell responses to mild and severe hyperthermia (39 and 45°C), and also mild and severe hypothermia (35°C and 27°C), the cells were sub-cultured under 5% CO_2 at 37°C overnight and then transferred to a different incubator with the required pre-adjusted temperature. The Osteoblast-like osteosarcoma cells MG-63 were used at passage 92 to 94.

Cell viability assay

The MTS assay was used to determine the cell viability of osteosarcoma cells under hyper- and hypothermia [20]. Briefly the cells were cultured with a seeding density of 5 x 10^3 cell/ well in 96 wells plate with 100 µL of complete culture media and were incubated overnight at 37°C and 5% CO_2 in a humidified incubator. After that the plates were transferred to a different incubator with pre-adjusted temperatures of 45°C, 39°C, 35°C and 27°C while control plates were left at 37°C. Cells were treated for 1, 12, 24 and 72 h. After that 20 µL of MTS (Promega, Madison, USA) was added per well and the plates were incubated for 4 h at 37°C. The absorbance was measured with a microplate reader (Biomeks FX Beckman Coulter, Brea, USA) at 490 nm.

DNA Damage

DNA damage was determined by single cell gel electrophoresis (SCGE) comet assay [21]. Briefly the cells were harvested with accutase (Life Technologies, Grand Island, USA) and washed once with ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS) and spun at 125 g for 5-10 mins. After washing the cells, the cells were re-suspended in DPBS. 100 µl of 1 % pulse field agarose (Bio-Rad, Hercules, CA, USA) in Dulbecco's Phosphate-Buffered Saline (DPBS) was boiled and added to fully frosted microscopy slides, covered with a coverslip and left to solidify in an ice box for 5-10 mins. Once the agarose was ready, the coverslips were removed and the cells were mixed with 100µL of 1 % low melt agarose (Bio-Rad, Hercules, CA USA) at 37°C. Another coverslip was used to distribute the cells equally and the slides were left in the ice box for 5 mins. After that the coverslip was removed and the slides were immersed in pre-chilled lysis solution (2.5 M NaCL, 100 mM Na_2 EDTA $2H_2O$, 10mM Tris base, 1% Triton X-100 and 10% DMSO (Sigma-Aldrich, St. Louis, MO, USA)) for one h at 4°C. Slides were taken out, rinsed 3 times in cold distilled water (dH2O) and located in electrophoresis horizontal tank (Bio-Rad, Hercules, CA, USA). Fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH>13) was added until the slides were covered and then left for 20-40 mins to allow the unwinding of DNA. The electrophoresis was run for 20 mins at 25 V and 300 mA. Slides were removed and washed 3 times with neutralization buffer (0.4 M Tris, pH 8.5) for 5 mins. Slides were then allowed to dry and stained with 0.01% CYBR green (Invitrogen-Molecular Probes, Foster, CA, USA) in TBE buffer. Slides were washed and covered with a coverslip. Hydrogene peroxide (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control at 100 µM (Supplementary, Fig. 1). All these steps were performed in dimmed light to avoid DNA damage that may result from white light. Slides were scanned using a florescent microscope connected to a digital camera (Olympus Co, Tokyo, Japan) and images were captured at 10x magnification. At least three replicates were run for every group. For the statistical analysis, one hundred cells were scored in every replicate.

Flow cytometric measurement of apoptosis using annexin v/propidium iodide (PI) staining

Annexin V-FITC kit from Beckman Coulter (France) was used to detect apoptotic cells. MG63 cells were seeded at a density of 1x10^4/cm in T-25 Flasks (Orange Scientific, Belgium), allowed to attach overnight and then exposed to hypo- and hyperthermia at various time points. At the end of the treatment, the cells were washed two times with ice-cold PBS (Phosphate-buffered saline), detached from the cell culture flask with the help of accutase and then centrifuged for 5 min at 500xg at 4°C. The supernatant was removed and the cell pellets were re-suspended in ice–cold 1x binding buffer to 5 x 10^5 ~ 5 x 10^6 cells /mL. 100 µL of cell suspension were stained with 1 µL of Annexin V-FITC solution and 5 µL of PI for 15 min and incubated on ice in the dark. A flow cytometer (Becton Dickinson, San Jose, CA, USA) was used for analyzing the cells [22].

Caspase activity

The activity of the caspases 3/7, 8, and 9 were estimated using a luminescence-based assay from Promega (Madison, Wisconsin, USA) [23]. Cells were seeded at a density of 5x10^3 cells/100 µL per well in white 96-well plates (SPL Life Sciences, Pocheon, Gyeonggi-do, Korea) and left to attach overnight at 37°C. At the end of the treatment with hypo- and hyperthermia, 100 µL of caspase reagent was added. The luminescence signal was measured using a plate reader (Perkin Elmer, Waltham, MA USA).

Protein expression of heat shock proteins 70, 27 and 90

Protein expression of Hsp70, 27, and 90 was measured according to the manufacturers' instructions, using a commercial ELISA kits from Abcam (Cambridge, England, UK), Assaypro (Missouri, USA), and eBioscience (San Diego, CA,USA), respectively. In brief, cells were harvested after treatment with hypo- and hyperthermia using accutase. The cell lysates were then washed twice with ice-cold PBS and centrifuged for 5 min at 500 x g at 4 °C. The cell pellets were lysed in 150 µL of extraction buffer. A protease and phosphatase inhibitor cocktail from Fisher Scientific (USA) was added to the extraction buffer. The protein quantity was estimated using the NanoDrop 2000 c Spectrophotometer (Thermo Scientific, USA) [24]. Colorimetric detection was determined by reading the absorbance at 450 nm (Perkin Elmer, Waltham, MA, USA).

Gene Expression

Gene expression was determined with real time PCR (Bio-Rad, USA), as described [17]. For cDNA synthesis 1 µg total RNA was used with iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, USA)) according to the manufacturer's instructions. The obtained cDNA was

diluted 5 times and 2 µL was used for expression of Hsp90 in a cocktail of 1x iQ™ SYBR Green Supermix (Bio-Rad). GAPDH and HPRT1 were used as a reference gene. The primer sequences used for the amplification of Hsp90, GAPDH and HPRT1 are shown in table 1.

Table 1 Primers sequences

Gene	Priemr	Sequence
Hsp90	Forward	5'-CCGGTGTAGGAATGACCAGAG-3'
	Reverse	5'-TGTCCCAGATTTGGCTATGGTA-3'
GAPDH	Forward	5'-TGCACCACCAACTGCTTAGC-3'
	Reverse	5'-GGCATGGACTGTGGTCATGAG-3'
HPRT1	Forward	5'-TGACACTGGCAAAACAATGCA-3'
	Reverse	5'-GGTCCTTTTCACCAGCAAGCT- 3'

PCR gene expression PCR array (RT^2 Profiler)

The RNeasy Mini Kit with on column DNase treatment step was used for RNA extraction. The step of genomic DNA elimination was carried out by mixing 2 µL of DNA Elimination buffer (Qiagen, Hilden, Germany) with 1-8 µL of total RNA. The mixture was incubated at 42°C for 10 minutes. For cDNA synthesis the RT^2 First Strand Kit (Qiagen, Hilden, Germany) was used. Each reaction containing 1µg of DNA-free RNA (10µL), l µL Primer and external control mix, 4 µL 5x RT buffer, 3 µL dH_2O and 2 µLRT enzyme mix (Qiagen, Hilden, Germany). This mixture were incubated in a thermal-cycler for 15 mints at 42°C and stopped by incubating at 95°C for a period of 5 minutes. Prior to running the PCR array, 91 µL of dH_2O was added to the cDNA and mixed gently (17).

Twenty six µL of the obtained cDNA was loaded into RT^2 Profiler after mixing with RT^2 PCR master mix (total volume 25 µL) (SABioscience) 96-well PCR array plates (CAPH10449) and amplified by using iQ™ 5 Real Time PCR detection system (Bio-Rad, USA). For data analysis the SABioscience (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) was used to calculate the p values and fold change. Gene expression was normalized to the expression of GAPDH (Catalog Number: PPH00150) and HPRT1 (Catalog Number: PPH01018) as reference genes using the RT^2 Profiler PCR Array.

Statistical analysis

All data were tested for normality. Statistical analysis was performed by Student's t-test for PCR array data and Microsoft Excel 97, 2003. For other experiments data were expressed as mean ± standard deviation and statistical analysis was performed by Student's t-test and SPSS version 17 (SPSS Inc, Chicago, Illinois, USA). A global significance level of $p < 0.05$ was used for all analyses.

RESULTS

Severe hyper- and hypothermia showed significant reduction in cell viability

MG-63 cells treated with severe hyper- and hypothermia showed significant reduction in cell viability as early as 1 h of treatment. Severe hyperthermia reduced cell viability to 30% and below from 12 h onwards. Treatment with mild hyperthermia did not show any effect on cell viability until after 24 h incubation at 39ºC. Then a significant reduction in cell viability was observed (Fig 1a). Severe hypothermia reduced cell viability at all time points. Mild hypothermia, in contrast, increased MG-63 cell viability to 120% after 12 h of incubation at 35ºC, which dropped below 100% after 24 h, but came up again to 112% after72 h (Fig. 1b).

Mild and severe hyperthermia-induced DNA damage in MG-63 human osteosarcoma cells

Human osteosarcoma MG-63 cells treated at 45°C showed DNA damage as early as 1 h of incubation (Fig. 2a). 20% comet positive cells were detected. After 12 h of incubation at 45°C, 100% DNA damage was observed. This result is consistent with the flow cytometry results in Fig. 3, which showed that 97% of the cells were apoptotic at 12 h and above. Mild hyperthermia showed slight DNA damage at all time points, which showed no significant differences compared to control. On the other hand, severe hypothermia showed significant increments of damaged cells at 72 h only, whereas mild hypothermia showed no significant differences with control (Fig. 2b).

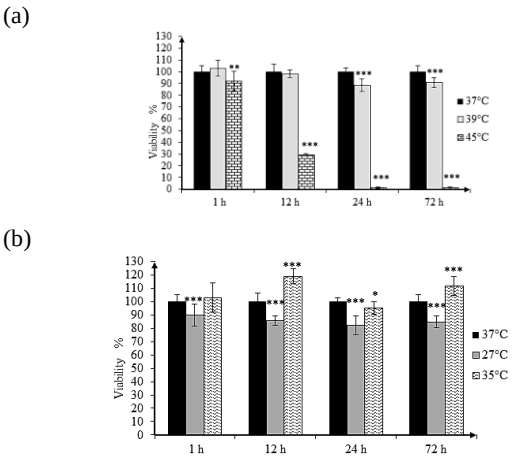


Fig. 1 Hyper- and hypothermia regulate viability in MG-63 osteosarcoma cells (a & b respectively). Shown are viability data after 1, 12, 24, and 72 h of treatment. Results represent the means of percentage change relative to control (100%) ± SD of three replicate samples (n=3). Student's t-test test was used and significance was set at *p< 0.05, ** p <0.01, and *** p <0.001

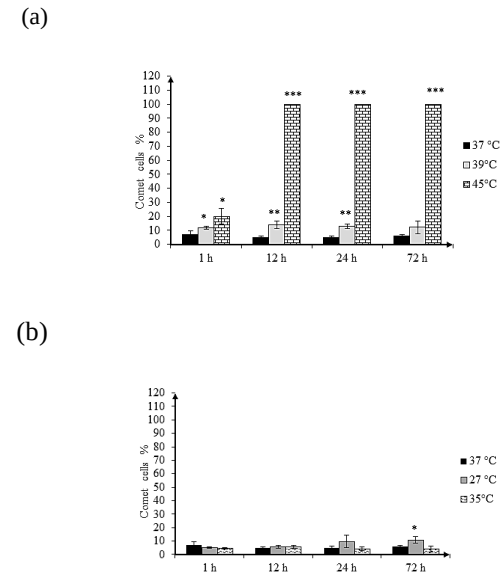


Fig. 2 DNA damage was measured after exposure to mild and severe hyper and hypothermia for 1, 12, 24, and 72 h (a & b respectively). Alkaline comet assay was used to evaluate DNA damage. The percentage of comet cells was scored for 100 cells. At a minimum, three independent experiments were averaged, and data are presented as the mean ± SD. Student's t-test was used to calculate the significance of the data, at *p<0.05, **p<0.01, and ***p<0.001

Apoptotic effect of mild and severe hyper- and hypothermia on MG-63 human osteosarcoma cells using Flow cytometry

MG-63 Cells were incubated with Annexin V-FITC in a buffer containing propidium iodide (PI) and analyzed by flow cytometry. Severe and mild hyperthermia treatment induced apoptosis in a significant manner at all time points (Fig. 5a,c,e and g). Especially severe hyperthermia caused a highly significant rate of apoptosis after 12 h and above ($p<0.001$). Severe hypothermia induced significant cell death after 24 and 72 h only. Mild hypothermia did not induce any apoptosis at any time points compared to untreated control (Fig. 3b,d,f and h).

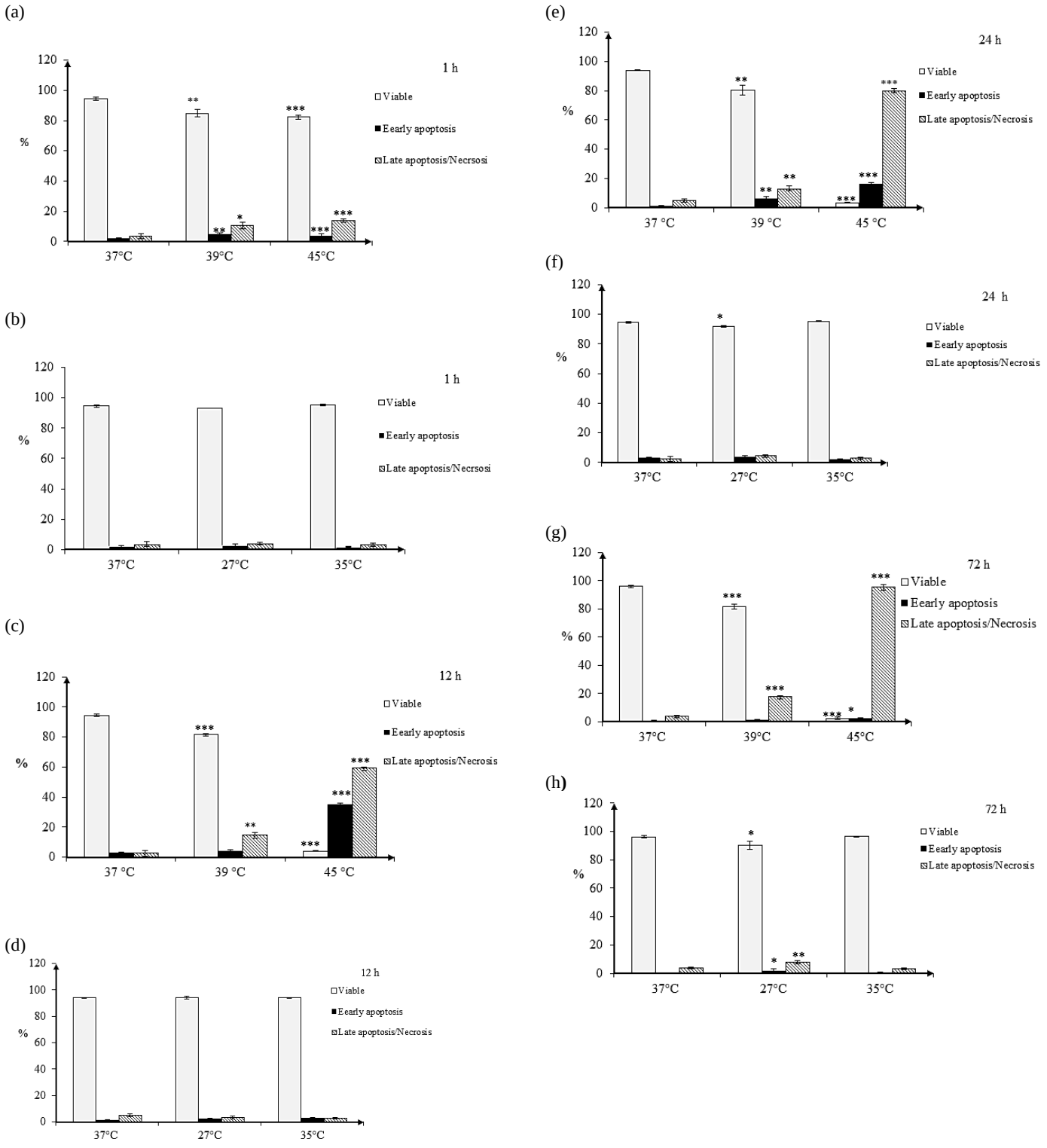


Fig. 3 Effect of hyper- and hypothermia on MG-63 cell apoptosis. Apoptosis of MG63 cells were determined using annexin V/PI staining after 1, 12, 24 and72 of hyperthermia (a,c,e and g) and hypothermia (3b,d,f and h) treatment. Data are expressed as mean ± SD of 3 biological replicates. Significance was determined using Student's t-test and set at Effect of mild and severe hyper- and hypothermia on the gene expression and enzyme activity of caspases 3, 8 & 9 in MG-63 human osteosarcoma cells*p<0.05, **p<0.01, and ***p<0.001.

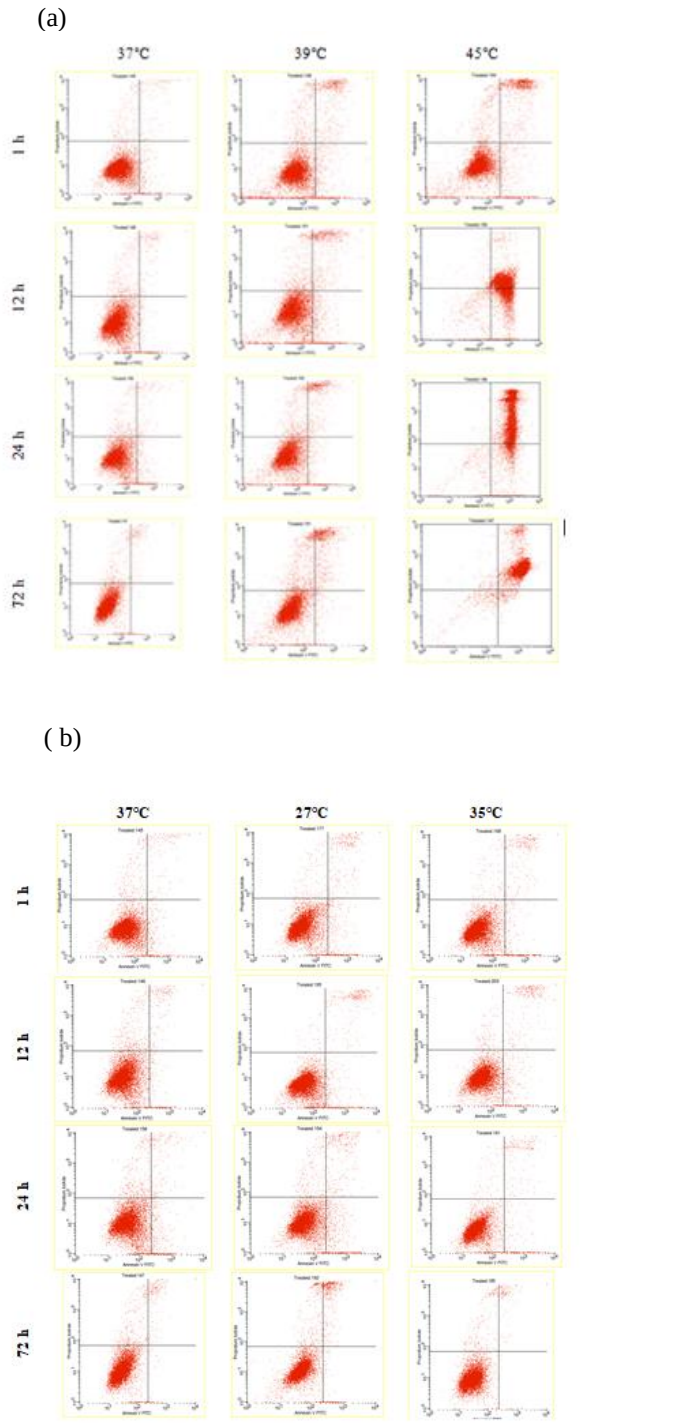


Fig. 4 Contour Diagram of Annexin V/PI Flow Cytometry. The lower-left quadrant indicates viable cells, excluding PI and negative for Annexin V binding. The upper-right quadrants contain non-viable necrotic cells and late apoptotic positive for Annexin V and PI uptake. Lower-right quadrants represent early apoptotic cells, Annexin V positive, and PI negative. Figure 4a shows data for hyperthermia (39 and 45°C) , while figure 4b shows data of hypothermia groups (27 and 35°C).

Effect of mild and severe hyper- and hypothermia on the gene expression and enzyme activity of caspases 3, 8 & 9 in MG-63 human osteosarcoma cells

Figure 4 shows the effect of hyperthermia on caspase 3, 8 and 9 gene expression and enzyme activities. Caspase 3 showed no significant changes in gene expression for mild hyperthermia while the gene and prtoein expressions for severe hyperthermia within 12-72 h couldn't be determined due to RNA degredation. No significant changes in caspase 3 activity were observed under mild hyperthermia while under severe hyperthermia the activity was significantly increased after 1 h followed. In contrast to caspase 3, caspase 8 showed a steady and significant increase in mRNA expression under mild hyperthermia. The peak of caspase 8 mRNA expression was reached at 24 h of incubation after which the expression dropped to control levels. The activity data did deviate from the gene expression data for 1 and 24 h. For severe hyperthermia no gene expression data could be obtained at 12 h and above. Caspase 9 showed a significant increase in gene expression and enzyme activity after 12h of mild hyperthermia.

Fig. 5 shows the effect of hypothermia on the gene expression and enzyme activities of caspase 3, 8 and 9. After 1 h of treatment, caspase-3 and 9 mRNA were decreased in all groups of mild and severe hypothermia. Additionally, caspase-8 mRNA was reduced significantly at 1 h under severe hyperthermia. Cells showed no significant difference in gene expression levels of caspase-3, 8, and 9 compared to negative control after 12 h of mild hypothermia. After 24 h of severe hypothermia, a significant reduction in the expression of caspase-3 and 8 was observed. Caspase-9 showed a slight but not significant reduction in mRNA expression. On the other hand, mild hypothermia significantly decreased the expression of caspase-3 and 8 transcripts ($p<0.05$). 72 h of severe hypothermia significantly decreased the expression of caspase-8 while the expression of caspase-3 only increased slightly. The expression of caspase-9 decreased in the meantime. Mild hypothermia decreased expression of caspase-3, 8, and 9.

Treatment with severe hypothermia for 1 h shows no caspase-3 or 8 activity. At 12 h the activity of all caspases was reduced in a significant manner. Severe hypothermia reduced the activity of all caspases at 24 h; especially that of caspase-3/7. Severe hypothermia induced caspase-3/7 activation at 72 h post treatment to a significant extent, while caspase-8 and 9 activities declined significantly.

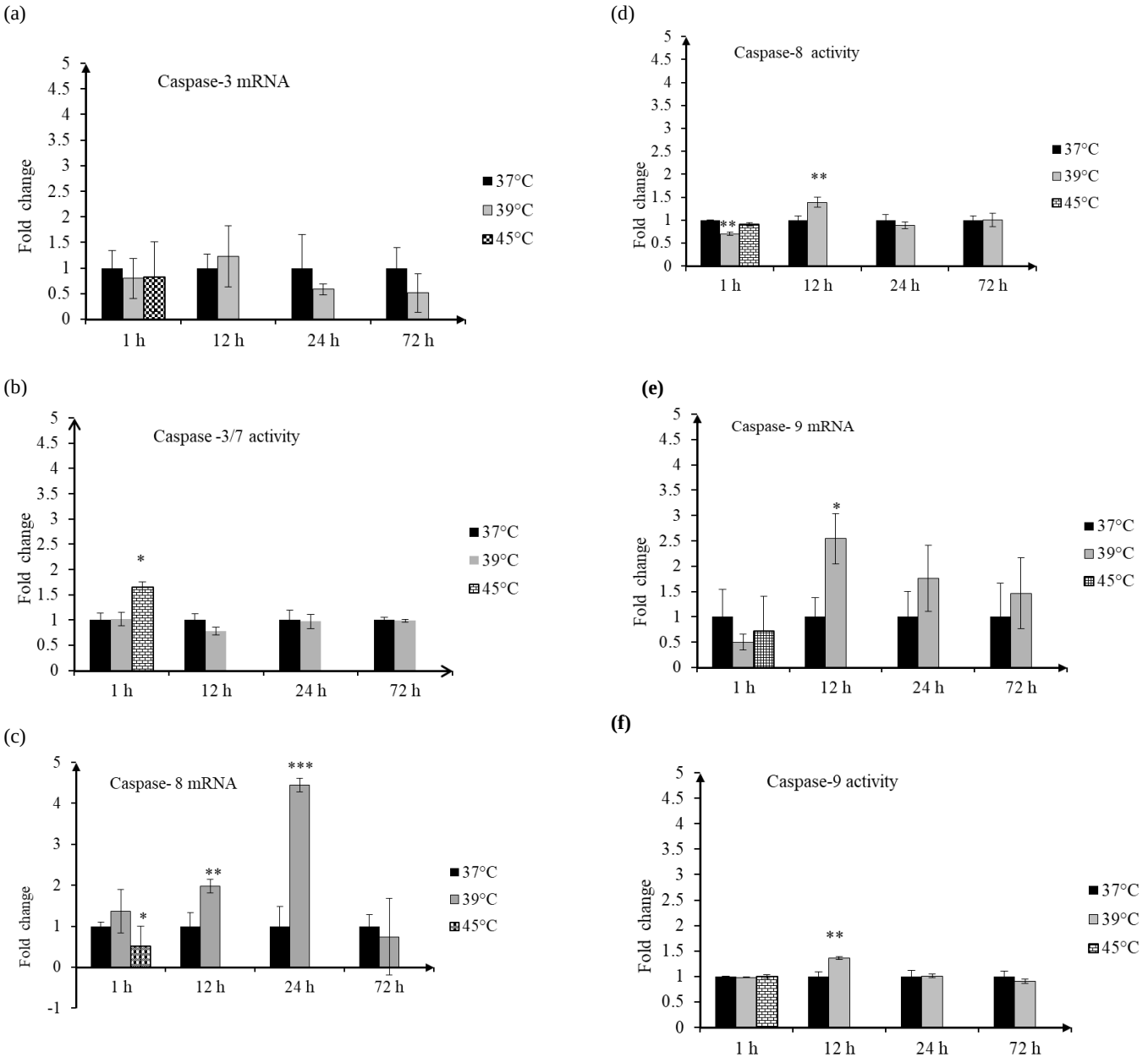


Fig. 4 Effect of hyperthermia on the gene expression and enzyme activity of caspases 3, 8 and 9 in MG-63 cells after exposure to mild and severe hyperthermia. Analysis was performed by RT^2 Profiler PCR Array and Caspase-Glo assay kit. Data are expressed as means (n=3). Statistical analysis done by Student's t-test showed significant differences at *p< 0.05, ** p <0.01, and *** p <0.001.

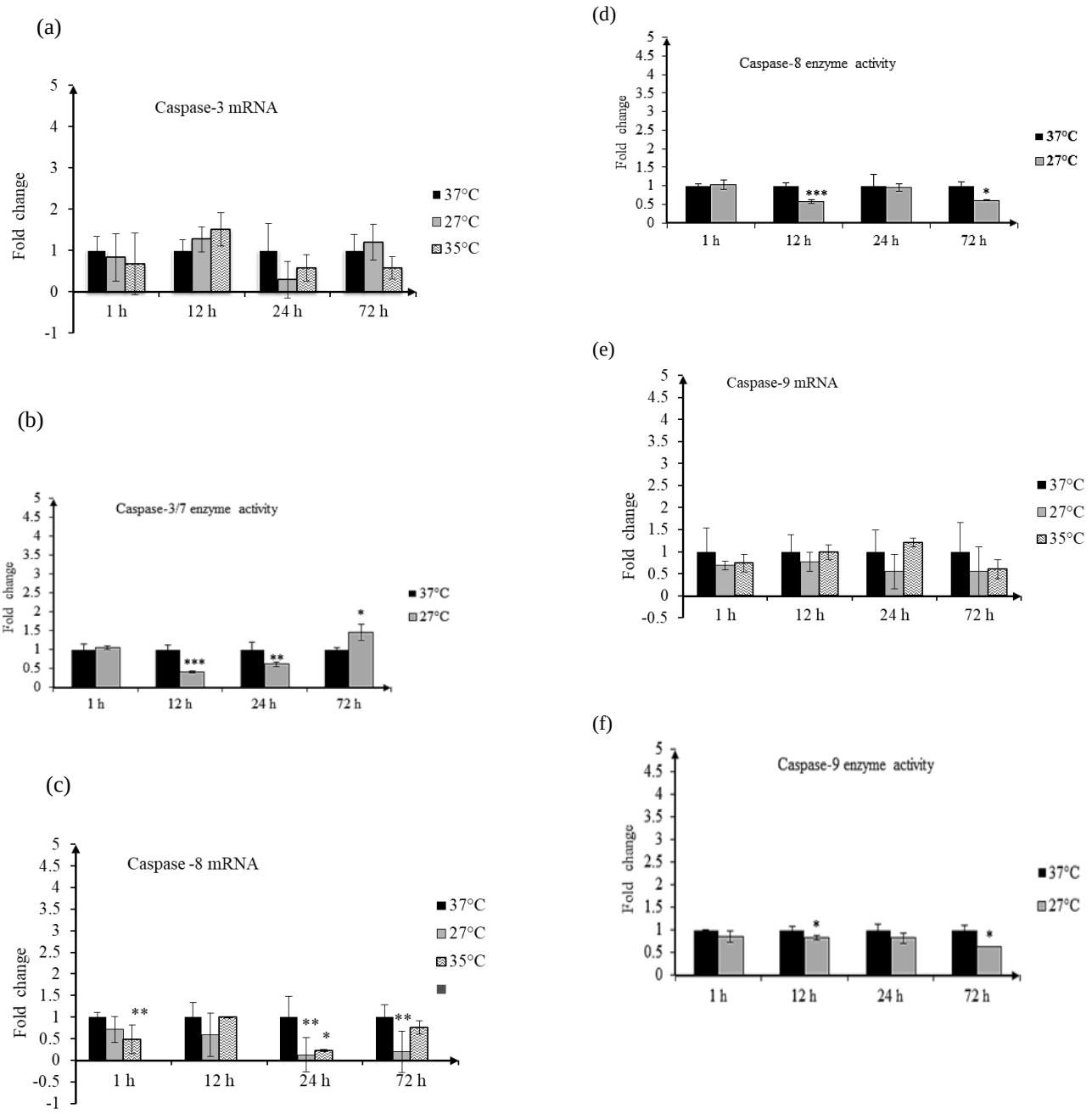


Fig. 5 Effect of hypothermia on the gene expression and enzyme activity of caspases 3 (**a** & **b**), 8 (**c** & **d**) and 9 (**e** & **f**) in MG-63 cells after exposure to mild and severe hypothermia Analysis was performed by RT^2 Profiler PCR Array System and Caspase-Glo assay kit. Data are expressed as means (n=3. Statistical analysis done by Student's t-test showed significant differences at *p< 0.05, ** p <0.01, and *** p <0.001.

Expression of heat and cold-shock proteins in MG-63 cells treated with mild and severe hypo- and hyperthermia

Fig. 6 and 7 show the gene and protein expression of heat and cold-shock proteins after treatment with mild and severe hyperthermia. Significant changes in the gene expression of Hsp90 were observed after 12 (downregulation), 24 (downregulation) and 72 h (upregulation) of mild hyperthermia treatment. The same trend was observed in the protein expression. From 12 h onwards, no gene and protein expression data for severe hyperthermia could be collected because of RNA degradation. Under mild hyperthermia the expression of Hsp70 mRNA showed a significant increase only after 12 h of incubation ($p<0.05$). On the other hand, protein levels of Hsp70 increased significantly after 24 and 72 h of treatment with mild hyperthermia ($p<0.01$ and $p<0.01$), whereas severe hyperthermia showed no detectable amount of Hsp70 from 12 h onwards due to RNA and protein degradation. Hsp27 mRNA levels increased in a significant manner ($p<0.01$) at 24 h of incubation in mild hyperthermia and were slightly up-regulated at other time points. Protein levels of Hsp27 showed slight increment at all time points of mild hyperthermia.

Mild hyperthermia induced a reduction in levels of RBM3 mRNA at all time points except for the 1 h group. On the other hand, CIRBP was reduced to a significant extent at 1 h. CIRBP mRNA significantly increased at 24 h (3.3-fold, $p<0.01$). Severe hyperthermia reduced CIRBP expression in a significant manner at 1 h ($p<0.01$) (Fig. 7).

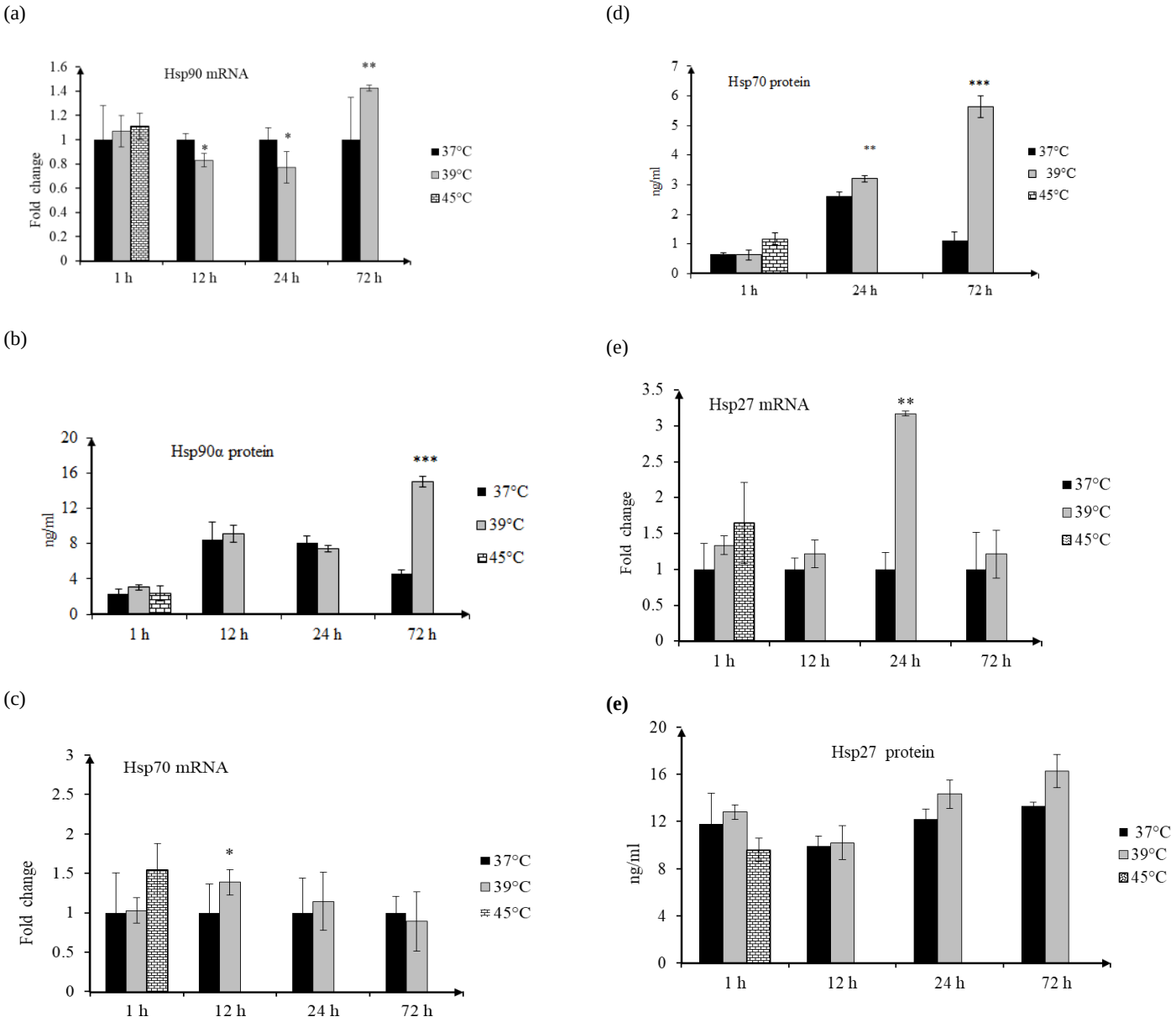


Fig. 6 Gene and Protein expression of Hsp90 (**a** & **b**) Hsp70 (**c** & **d**) and Hsp27 (**e** & **f**) after exposure to mild and severe hyperthermia for 1, 12, 24 and 72 h. PCR array was used to measure the gene expression and data represent mean fold change. Protein expressions were measured using ELISA and represent mean fold change (n=3) ± SD. Statistical analysis done by Student's t-test showed significance at *p< 0.05, ** p<0.01, ***p<0.001.

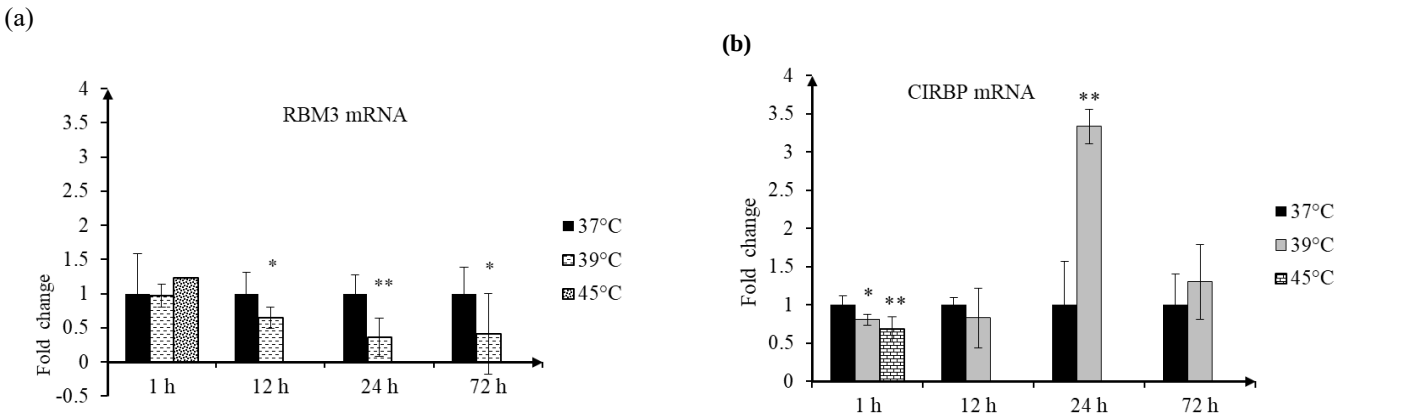
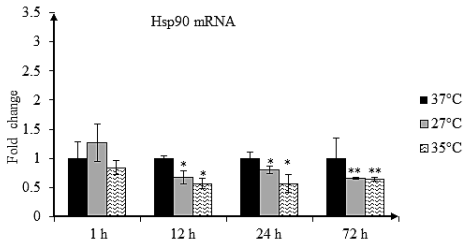


Fig. 7 Gene expression of RBM3 (**a**) and CIRBP (**b**) mRNA in MG-63 cells after exposure to mild and severe hyperthermia for 1, 12, 24 and 72 h. PCR array was used to measure the gene expression. Data represent mean fold change (n=3. For statistical analysis Student's t-test was used and significance was set at *p< 0.05, ** p<0.01

A significant downregulation of Hsp90 mRNA expression was observed after 12, 24, and 72 h incubation of MG-63 cells in hypothermia (Fig. 8). Under severe hypothermia, Hsp70 mRNA levels were reduced at 24 and 72 h to less than half compared to the negative contrgol. At 1 h it showed a slight upregulation, whereas no difference was seen at 12 h. Mild hypothermia suppressed Hsp70 mRNA in a significant manner at 12 h ($p<0.05$) only. Under severe hypothermia Hsp27 transcript was reduced at 12 and 24 h ($p>0.05$), while a slight increase was observed at 1 and 72 h compared to untreated controls. Mild hypothermia induced a significant decrease at 24 h ($p<0.01$) only (Fig. 8c).

After hypothermia treatment, MG-63 showed an upregulation of the cold-shock proteins RBM3 and CIRBP (Fig. 9). RBM3 mRNA was significantly upregulated after 1 and 72 h of mild hypothermia ($p<0.05$ and 0.01), while CIRBP was significantly upregulated after 1 and 12 h of mild and severe hypothermia (Fig. 8). After 24 h CIRBP showed a significant increase after mild hypothermia ($p<0.01$), but a decrease in expression under severe hypothermia. After 72 h of mild and severe hypothermia, the expression of CIRBP returned to control levels.

(a)



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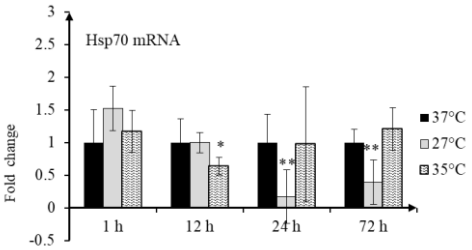


(c)

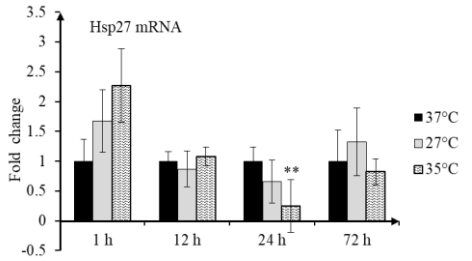
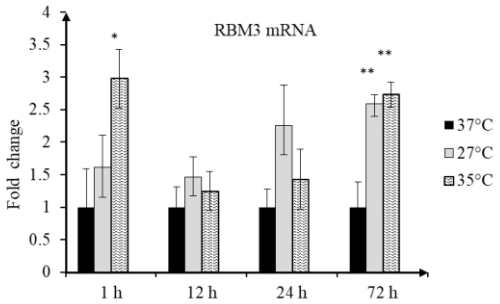


Fig. 8 Gene expression of Hsp90 (**a**) Hsp70 (**b**) and Hsp27 (**c**) after exposure to mild and severe hypothermia for 1, 12, 24 and 72 h. PCR array was used to measure the gene expression and data represent mean fold change (n=3). For statistical analysis Student's t-test was used and significance was set at *p< 0.05, ** p<0.01, ***p<0.001.

(a)



(b)

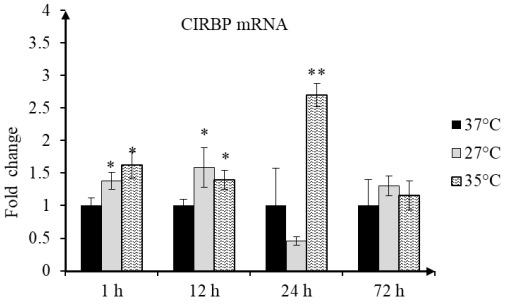


Fig. 9 Gene expression of RBM3 (**a**) and CIRBP (**b**) in MG-63 cells after exposure to mild and sever hypothermia for 1, 12, 24 and 72 h. PCR array was used to measure the gene expression. Data represent mean fold change (n=3). For statistical analysis Student's t-test was used and significance was set at *p< 0.05, ** p<0.01

DISCUSSION

Severe hyperthermia especially showed massive cellular death at 12 h and above in this study. Accordingly, MTS assay revealed that severe hyperthermia reduced cellular viability, especially at 12 h and above. The majority of cells were in a stage of late apoptosis, which could be attributed to acute and prolonged exposure to hyperthermia. Garcia et al. (2012) found that acute hyperthermia caused an increased rate of cell death in late apoptosis [25]. An additional observation was that hyperthermia caused severe cell detachment due to changes in the cytoskeleton of the osteosarcoma cells (unpublished data). This form of cell death is called anoikis. Luchetti et al. (2004) observed that hyperthermia induced cytoskeletal changes, cell rounding accompanied by surface blebbing, and anoikis of neuroblastoma cells [26]. They concluded that these are early symptoms of hyperthermia-induced apoptosis. However, the detachment of cells was less dramatic than observed in osteosarcoma cells. The overall reduction in cell viability caused by mild hyperthermia was significantly less dramatic than the one resulting from severe hyperthermia. Looking at cell viability results and the phenomenon of cell detachment, one could speculate that mild and severe hyperthermia induces apoptosis differently. O'Neill et al. (1998) were able to show that exposure to even mild hyperthermia induces apoptosis and that increasing the temperature and duration shifted the mode of cell death from apoptosis to necrosis [27]. Several other studies reported that hyperthermia reduces the viability of several cancer cell lines, such as melanoma cells, osteosarcoma cell HOS85, MG-63 and SaOS-2 , human breast cancer (MDA-MB 231), and a non-small cell lung cancer cell line (H1299) [9,28-30]. Osteosarcoma cells were treated with severe hypo- and hyperthermia for 12 hours and above to study the molecular responses underlying these conditions. Currently, the obtained data from these groups are of fundamental value more than any clinical relevant or applications.

Mild hypothermia (35°C) reduced the cellular viability of osteosarcoma cells in a significant manner after only 24 h, while a 12 and 72 h treatment increased mitochondrial activities. In agreement with these results, normal osteoblast cells showed an increased

mitochondrial activity after 72 h under mild hypothermia [17]. Interestingly, another study showed that hypothermia protects normal cell while creases sensitivity of some tumour cells against anti-tumor drug 5-fluorouraci [31]. An earlier study on the small bowel and colon of rats that had received azoxymethane showed that mild hypothermia induced cell proliferation [32]. So far, no studies using hypothermia on osteosarcoma cells could be found. However, it is well-known that mild hypothermia is utilized to increase cellular activities for producing recombinant proteins in the field of biotechnology [33,34]. On the other hand, severe hypothermia showed a time-dependent toxicity, whereby viability was reduced in a significant manner at all time points. An earlier study reported that severe hypothermia (25°C) for 48 h affects cell proliferation by about 95%, and leads to accumulation of cells at G2 [35]. In agreement with the findings of this study, Aisha et al. (2014) found that severe hypothermia (27°C) resulted in the reduction of osteoblast cell viability [17]. This study shows that severe hypothermia not only reduces cell viability of MG-63 cells in a dose- and time-dependent manner, but also causes apoptosis.

For additional understanding of the anticancer effect of hyperthermia, the comet assay was used to determine DNA strand breaks in MG-63 cells. Severe hyperthermia caused a significant amount of DNA damage at all time points. The causes for the observed DNA damage are double-strand breaks (DSB), which also see the enhanced formation of H2AX foci [36]. Severe hyperthermia suppressed the repair of DNA damage induced by UV in HeLa cells [37]. An additional factor in the survival of cancer cells with DNA damage is the role of heat-shock proteins. In the present study, severe hyperthermia showed significant DNA damage at all time points, whereas mild hyperthermia showed significant genotoxicity at all time points except at 72 h, which may be attributed to the significant induction of Hsp90.

Mild hypothermia did not show any genotoxicity when tested for deoxyribonucleic acid (DNA) damage after the treatment. This provides an extra evidence of the potential cytoprotective role of mild hypothermia. Severe hypothermia, on the other hand, showed DNA damage at 72 h. Mild hypothermia did not induce cell death, which is confirmed by comet assay indicating the protective activity of mild hypothermia. On the other hand, severe hypothermia induced apoptosis at 24 h and more. This is the first report about the effect of hypothermia on osteosarcoma cells. Kalamida et al. (2015) found that hypothermia induced caspase-9 in DU145, MCF7, and U87MG cells in association with reduced viability [5].

Caspase-3 was activated in the severe hyperthermia group, which was treated for 1 h and severe hypothermia 72 h, but no caspase-8 or 9 activation was shown at these time points. In contrast to this finding, Kalamida et al. (2015) found hyperthermia- and hypothermia induced caspase-9 in DU145, U87MG, and MCF7, in association with viability reduction [5]. However, the classical pathway of apoptosis (caspase-dependent) cannot be ruled out here. Milhas et al. (2005) mention that caspase-10 plays a role in this pathway [38]. They found that caspase-10 triggers Bid cleavage and caspase cascade activation in FasL-induced apoptosis [38]. One hour of severe hyperthermia led to a reduction of Bid protein expression. However, there was no change in FasL expression (unpublished data from Nashiry et al.). It is, of course, possible that 1 h exposure to severe hyperthermia is not sufficient to see changes at the protein level. Mild hyperthermia induced increased expression of caspase-8 and 9 mRNA after 12 and 24 h. This was mirrored when measuring the activity of caspase-8 and 9, indicating that both pathways (extrinsic and intrinsic) were equally activated by mild hyperthermia.

Heat-shock proteins are molecular chaperones that aid in the stabilization and folding of proteins [39]. Exposure of MG-63 cells for 12 and 24 h to mild hyperthermia resulted in a reduction in Hsp90 mRNA, while there was no significant change in protein expression. This shows that while mild hyperthermia affects the transcriptional level, it has no effect at the translational level. In contrast to that, 72 h of mild hyperthermia induced a significant upregulation of Hsp90 mRNA and protein, indicating the development of possible heat resistance. However, since Hsp90 does not act alone, the results need to be seen in conjunction with changes happening to Hsp70 and Hsp27. The increment of Hsp90 for mild hyperthermia could contribute to decreased DNA damage at 72 h.

Hsp27 aids in maintaining the integrity of mitochondria and inhibition of caspase activation, because it blocks the release of cytochrome c [40]. Concannon et al. (2001) found that Hsp27 inhibits caspase-3 processing [41]. On the other hand, it was reported that overexpression of Hsp27 suppresses tumor migration under hyperthermia conditions [42]. Mild hyperthermia induced gene expression in a significant manner in the current study. The protein expression of Hsp27 at 24 h was not consistent with gene expression. On the other hand, a previous study demonstrated that Hsp70 expression had been associated with good response to chemotherapy in osteosarcoma [43]. Hyperthermia caused Hsp70 upregulation and viability reduction of osteosarcoma cells [9], which is consistent with our study since severe hyperthermia (1 h) caused upregulation of Hsp70 at both translational as well as transcriptional levels.

Long-term exposure (12-72 h) of MG-63 cells to mild and severe hypothermia showed a significant reduction in Hsp90 gene expression. Hsp70 gene expression was less susceptible to mild hypothermia, but severe hypothermia reduced its level significantly at 24 and 72 h. A recent paper from Kalamida et al. (2015) indicates that exposure to mild hypothermia (34°C) causes a reduction of Hsp90 protein expression in various cancer cell lines, like lung and prostate adenocarcinoma [5]. The authors speculate that hypothermia could be a physical agent to reduce tumor growth, or it could act as an adjuvant to chemotherapy. Considering that severe hypothermia downregulates Hsp90 in osteosarcoma cells, this is a promising sign.

In our study, Hsp27 was slightly induced at mRNA levels at 1 and 72 h for severe hypothermia, but mild hypothermia showed higher increases (2.3 fold) at 1 h. Mild hypothermia for 24 h suppressed Hsp27. This reduction could reflect the cytoprotective role of Hsp27. Upregulation of Hsp27 in osteosarcoma patients at diagnosis was the strongest negative prognostic marker for the efficiency of chemotherapy in patients [44]. Therefore, any reduction in Hsp27 expression should improve the outcome of chemotherapy treatment. Morii et al. (2010) found that inhibition of Hsp27 eliminated the resistance of osteosarcoma cells against zoledronic acid treatment [45]. Taken together, severe hypothermia for 24 h reduced all investigated heat-shock proteins, and therefore, could be a potential adjuvant in the treatment of osteosarcoma.

CIRBP and RBM3 are cold shock inducible proteins reported to act as RNA chaperones and to regulate translation-transcription during hypothermia [46,47]. It is reported that knocking down the cold-shock proteins can enhance the sensitivity of tumor cells to chemotherapy [46,47]. Severe and mild hyperthermia (1 h) in the present study resulted in the downregulation of CIRBP, which may explain the significant cell death. Several studies have found that RMB3 expression and its nuclear localisation is associated with improved survival in many forms of cancer, such as ovarian cancer, breast cancer, malignant melanoma, and prostate cancer [48-50]. Mild hyperthermia showed the suppressive effect on RBM3, whereas CIRBP was upregulated in a significant manner at 24 h only. However, the estimation of the protein level is recommended to conclude the role of cold-shock proteins.

Mild hypothermia showed a significant increase in expression of CIRBP at all time points except at 72 h, whereas severe hypothermia caused significant increases at 1 and 12 h. The significant change in expression of CIRBP was associated with no apoptosis in case of mild hypothermia, which may indicate the protective role of CIRBP in these cells.

A link between RBM3 expression and increased sensitivity to cisplatin was observed in ovarian cancer cells [49]. In the present study, RBM3 was significantly induced at 72 h at both groups of hypothermia and 1 h at 35°C. Zeng et al. found that the overexpression of RBM3 in prostate (PC-3) cancer cells greatly attenuated the stemcell-like feature of the cancer cells [51]. Aisha et al. (2014) demonstrate that the induction of the RBM3 in osteoblast cells under hypothermia resulted in the stabilisation of mRNA, and in increased transcription of functional bone markers, such as alkaline phosphatase (Alp) and osteocalcin (OCN) [17]. Findings from both studies indicate that RMB3 promotes cancer cell differentiation and reduced cell proliferation.

However, the current study recommends estimation of the protein level in the role of cold-shock proteins.

CONCLUSION

This study showed that compared to mild hyperthermia and severe hypothermia, severe hyperthermia was more effective in inducing apoptosis, genotoxicity, and reducing cellular viability at all time points. Hypothermia need further studied to determine possibilities of its usage for the treatment and manipulation of cancer.

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