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*Original Article*

# Heat stress induces both apoptosis and necrosis in normal human osteoblasts without heat shock protein-60 (HSP60) release

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**Abstract**

Thermal trauma can irreversibly damage bone cells; however, the mechanisms by which thermal trauma affects the bone microenvironment are poorly characterized. Heat shock protein-60 (HSP60) can be induced by stresses, including hyperthermia, and released from cells as an endogenous danger signal. The aim of this study was to determine the effects of heat stress on HSP60 release by human osteoblasts. Normal human osteoblasts (NHOst) were exposed to heat stress at 40°C to 46°C for 5-15 min and then cultured for 24 h. Cell viability was analyzed using the MTT assay. HSP60 protein expression and release were analyzed by Western blotting of cell lysates and conditioned medium. HSP60 subcellular localization was analyzed using immunocytochemistry. Annexin-V-FITC/propidium iodide staining and the lactate dehydrogenase (LDH) assay were used to investigate the mechanisms of cell death. We found that heat-stress significantly reduced NHOst cell viability in a dose- and time-dependent manner ($p<0.05$). Heat stress did not induce HSP60 protein expression or release by human osteoblasts; however, freeze-thawed necrotic human osteoblasts released HSP60 into the medium. Immunocytochemistry revealed modest changes in the subcellular localization of HSP60 in human osteoblasts after heat stress. Both apoptosis and necrosis were induced in human osteoblasts after heat stress. In conclusion, hyperthermia at temperatures as low as 43°C induced both apoptotic and necrotic cell death in human osteoblasts; however, heat treatment did not induce HSP60 protein expression or release into the extracellular milieu.

**Keywords:** heat stress, apoptosis, necrosis, HSP60, human osteoblast

## 1. Introduction

Surgical trauma, including thermal trauma, is one of the most common factors which contributes to failures in bone healing. Although great care is usually taken to avoid damage to bone tissues during surgery, in situations where there is a high bone density, surgeons may be at risk of overheating the bone. Thermal trauma, defined as a temperature above 47°C for more than 1 minute, can cause irreversibly bone cell damage or bone cell death, which leads to extensive bone resorption and failure of bone healing. Although cell stress and cell death normally occur after bone injuries, it is not well understood how cell stress and cell death affect bone healing. Sublethal stress can also exert a cytoprotective role and prevent cell damage in response to subsequent severe stress (Wheeler *et al*., 2007).

Mammalian species have developed numerous mechanisms to cope with stress. At a cellular level, these include the synthesis and function of heat shock proteins (HSPs), a major class of stress proteins such as HSP60, HSP70 and

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HSP90. Increased expression of HSPs can be triggered by a variety of stressful stimuli including elevated temperature (Diller, 2006). The unifying function of HSPs is chaperone activity, which contributes to cell survival during stress by facilitating the proper folding of denatured proteins (Hartl *et al*., 2002). In addition to their chaperone functions, HSPs can also exert anti-apoptotic or pro-apoptotic roles by interacting with other cellular proteins (Arya *et al*., 2007). For example, cytosolic HSP60 is anti-apoptotic as it binds to pro-apoptotic Bax protein in cardiac myocytes (Kirchhoff *et al*., 2002); whereas mitochondrial HSP60 binds to procaspase-3 to enhance apoptosis (Samali *et al*., 1999).

Until recently, stress proteins were considered to be exclusively intracellular; however, growing evidence suggests that stress proteins may also exist and function outside the cell. For example, tumor cell lines can release HSP60 into the culture media via an active mechanism which is independent of cell death (Merendino *et al*., 2010). Several studies suggest that cellular stress results in increased surface expression and release of stress proteins, which contributes to innate and adaptive immune activities (Bausero *et al*., 2005; Goh *et al*., 2011; Gupta *et al*., 2007). Extracellular HSP60 can induce chemotaxis, modulate neutrophil function, and stimulate phagocytosis (Goh *et al*., 2011; Osterloh *et al*., 2009). In addition, HSP60 has been shown to induce the maturation, cytokine release and T cell-activating capacity of dendritic cells (Flohe *et al*., 2003). Additionally, HSP60 can regulate T cell-mediated inflammation (Zanin-Zhorov *et al*., 2005) and also stimulates the production of a variety of proinflammatory mediators by innate immune cells (Habich *et al*., 2007). Recently, it has been reported that high concentrations of HSP60 act as a pro-inflammatory signal while low concentrations of HSP60 provides an anti-inflammatory signal to maintain immune equilibrium (Quintana *et al*., 2011).

Inflammation and the innate immune response play important roles in the initiation of bone healing. After bone injury, the hematoma and necrotic tissues related to bleeding begin to be cleared by cells associated with innate immune responses. Various inflammatory factors such as prostaglandins, interleukins, TNF-$\alpha$ and interferon-$\gamma$ are induced within the first 3 days after fracture (Barnes *et al*., 1999). In addition to their effects on immune cell function, these inflammatory cytokines also play key roles in initiation of the repair process by exerting direct effects on bone cells. For example, IL-6 induces alkaline phosphatase in MC3T3-E1 osteoblast-like cells (Cho *et al*., 2007), while TNF-$\alpha$ induces apoptosis in osteoblasts (Bu *et al*., 2003). Osteoblasts can also secrete proinflammatory cytokines including prostaglandin E2 ($PGE_2$) and IL-6 (Schmidt *et al*., 2003). Among several HSPs, such as HSP27, 70 and 90, only HSP60 has been reported to be increased in the plasma of postmenopausal women, however, the source of HSP60 remains unknown. Extracellular HSP60 has also been shown to directly increase osteoclastic bone resorption and osteoblast apoptosis *in vitro* (Koh *et al.*, 2009; Kim *et al.*, 2009; Meghji *et al*., 2003). This evidence suggests that HSP60 is an intracellular signal which may play a critical role in bone remodeling and bone healing.

Recently, the extracellular roles of HSPs in trauma-associated inflammation have begun to be appreciated. While some studies suggest that HSPs are released from dying, necrotic cells in a nonspecific manner (Basu *et al*., 2000; Saito *et al*., 2005), other researchers have shown that viable cells can release HSPs in a specific and inhibitable manner (Lancaster *et al*., 2004; Merendino *et al*., 2010). The release of HSPs from necrotic and/or stressed cells may serve as endogenous danger signals to stimulate macrophage and dendritic cells, thus promoting inflammation and regulating the innate and adaptive immune responses (Quintana *et al*., 2005; Zedler *et al*., 2006). While the role of extracellular HSPs, especially HSP60, in initiation and promotion of the inflammatory response to injury have become increasingly well understood, relatively little is known about the source of extracellular HSP60 and the mechanism by which it is released during injury or stress. In the present study, we hypothesized that heat stress could induce HSP60 protein expression and release, and investigated the potential mechanisms of heat-induced HSP60 release from human osteoblasts.

## 2. Materials and Methods

### 2.1 Cell culture

Normal human osteoblasts (NHOst) were obtained from Cambrex (Lonza, Walkersville, MD, USA). The cells were cultured in osteoblast growth medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), ascorbic acid and Gentamycin/Amphotericin-B at 37°C in a humidified atmosphere containing 5% $CO_2$. The culture medium was replenished every 2-3 days and the cells were subcultured once a week using Trypsin/EDTA (Invitrogen). For the heat stress studies, the cells were cultured until confluent, then, incubated in a thermostatic water bath at 40°C, 43°C or 46°C for 5 min, 15 min or 30 min. The control groups were incubated at 37°C under the same conditions. To mimic necrosis, NHOst cells suspended in medium were subjected to four freeze-thawed cycles in liquid nitrogen with thawing at room temperature.

### 2.2 MTT assay

Cell viability was analyzed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium salt (MTT) assay (Sigma-Aldrich, St.Louis, MO, USA). Briefly, 24 h after heat-stress treatment, the cells were replenished with RPMI-1640 phenol red-free medium (Invitrogen), MTT (5 mg/ml) was added to the medium and the cells were incubated at 37°C for 3 h. The formazan crystals were solubilized in acidic isopropanol, absorbance was measured at a wavelength of 570 nm and cell viability was calculated as percentage of untreated control cells.

### 2.3 Western blotting analysis of cell lysates and conditioned medium

Western blot analysis was performed following standard protocols. Briefly, the cells were lysed using lysis buffer containing 1% Triton-X and protease inhibitors. The total protein concentration was analyzed using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). To characterize the release of HSP60 from cells, the conditioned medium was collected and concentrated by freeze drying. The concentrated samples were reconstituted with equal volume of 2X SDS buffer, boiled for 5 min, and stored at -80°C until analysis. Western blotting was performed using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were incubated with a HSP60 rabbit polyclonal antibody (Chemicon, Billerica, MA, USA) followed by HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). β-actin (Cell Signaling Technologies, Danvers, MA, USA) was used as an internal control. The antibody signals were developed using SuperSignal West Pico substrate (Pierce), and the chemiluminescent signals were captured by autoradiography.

### 2.4 Immunofluorescence

The cells were fixed in 3.7% formaldehyde and permeabilized in 0.1% Triton X-100. Non-specific antigens were blocked by incubating the cells in 10% FBS for 30 min. Rabbit HSP60 polyclonal antibody (Chemicon) and FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc.) were used to detect the subcellular localization of HSP60. The slides were mounted in Fluoromount G mounting medium (SouthernBiotech, Birmingham, AL, USA), dried overnight at room temperature, and the immunofluorescent images were captured and analyzed using an inverted epifluorescence microscope.

### 2.5 Annexin V-Fluorescein/Propidium Iodide assay

The cells were stained with Annexin-V-fluorescein and propidium iodide (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. Briefly, the cells were incubated with an equal volume of Annexin-V-Fluos reagent and propidium iodide solution under coverslips for 10-15 min, and the apoptotic and necrotic cells were analyzed using an inverted epifluorescence microscope.

### 2.6 Lactate Dehydrogenase (LDH) assay

The conditioned medium was collected and LDH release was analyzed by using the Cytotoxicity Detection Kit$^{PLUS}$ (LDH) (Roche Applied Science, Indianapolis, IN, USA). Briefly, the conditioned medium was placed into 96-well plates and an equal volume of diaphorase/$NAD^+$ and iodotetrazolium chloride/sodium lactate reaction mixture was added. The reaction was incubated at room temperature for 30 min in the dark, stopped and analyzed by spectrophotometry at wavelengths of 492 and 690 nm. LDH release in each sample was calculated as a percentage of untreated control medium.

### 2.7 Statistical analysis

At least 3-4 samples were used for each experiment and each experiment was repeated twice. The results are presented as mean ± SE. Homogeneity of variance and statistical significance was analyzed using one-way ANOVA. The significance of the differences between the treatment groups and control group were analyzed using Dunnett's t multiple comparisons ($p$ values < 0.05 were considered significant).

## 3. Results

### 3.1 Heat stress decreases the viability of human osteoblasts in dose- and time-dependent manner

To determine the effect of heat stress on the viability of NHOst cells, we incubated the cells at 40°C to 46°C for 5 to 30 min and performed the MTT assay 24 h later. Exposure to heat stress at 40°C for 5 min, 15 min and 30 min had negligible effects on the cell viability of NHOst cells compared to control (Figure 1). However, cell exposed to 43°C or 46°C for 5 min, 15 min or 30 min had significantly lower cell viability than control cells ($p$<0.05). Phase contrast microscopy revealed slight changes in the cell morphology of heat-treated NHOst cells after exposure to heat stress at 40°C for 30 min, whereas cell shrinkage was observed after exposure to heat stress at 43°C and 46°C for 30 min.

### 3.2 Heat stress does not increase HSP60 protein expression or induce the release of HSP60 into the medium by human osteoblasts

To determine whether heat stress induces HSP60 protein expression in human osteoblasts *in vitro*, we performed Western blot analysis of NHOst cell lysates after

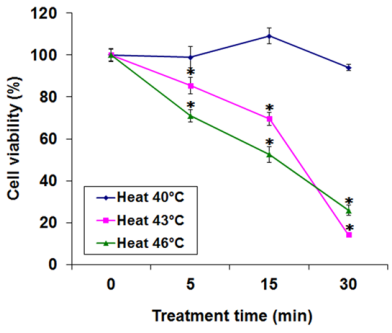


Figure 1. Exposure to heat stress reduces the viability of normal human osteoblasts. Cell viability was expressed as the mean ± SE percentage value of untreated control cells (100%). *, $p$<0.05 vs. untreated control cells.

exposure to heat stress at 40°C to 46°C for 5 to 30 min. We found that heat stress did not increase HSP60 protein expression in human osteoblast cells after heat stress treatments, compared to the control group incubated at 37°C (Figure 2A).

Given that most dying cells, especially necrotic cells, can release endogenous proteins which act as danger signals, we determined whether HSP60 was released from necrotic and heat stressed NHOst cells into the conditioned medium. After four freeze-thaw cycles, NHOst cells were non-viable and could be stained with trypan blue (data not shown). Interestingly, Western blot analysis revealed that only freeze-thawed necrotic cells, but not heat-stressed human osteoblasts, released HSP60 into the conditioned medium (Figure 2B).

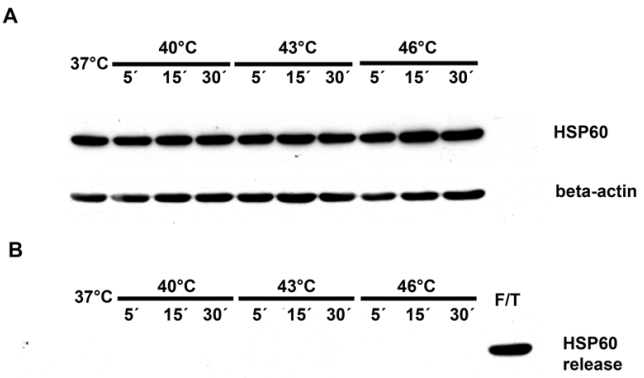


Figure 2. Heat stress does not increase HSP60 protein expression or induced the release of HSP60 into the medium by normal human osteoblasts. (A) Intracellular HSP60 protein expression in cell lysates. β-actin was used as an internal control. (B) HSP60 protein release into the conditioned medium. The conditioned medium of 4x freeze-thawed cells (F/T) was used as a positive control.

### 3.3 HSP60 subcellular localization is not affected by heat stress in human osteoblasts

As some studies have reported that HSP60 translocates from the mitochondria or cytosol to the plasma membrane during stress, we used immunocytochemical analysis to determine if the subcellular localization of HSP60 changed after heat stress. HSP60 was primarily localized to the mitochondria of control human osteoblasts. Heat stress treatment at 40°C to 46°C for 5-30 min did not alter the intracellular localization of HSP60 in NHOst cells. These results confirmed that HSP60 is mostly retained within the mitochondria of heat-stressed or lethally heat-treated NHOst cells (Figure 3).

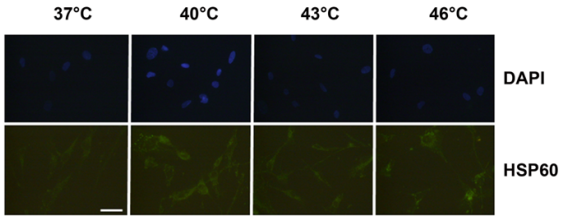


Figure 3. The subcellular localization of HSP60 is not affected by heat stress in normal human osteoblasts. The micrographs are arranged to show identical fields of DAPI nuclear staining (blue) and the HSP60 antibody staining (green). Scale bar = 50 μm.

### 3.4 Heat stress treatment induces both apoptosis and necrosis in human osteoblasts

We used Annexin-V-Fluorescein/propidium iodide immunocytochemical analysis to detect the presence of apoptotic and necrotic NHOst cells after heat treatment. Heat stress at 43°C or 46°C for 30 min induced both apoptosis and necrosis in human osteoblasts (Figure 4). To further characterize the extent of heat-induced necrosis or secondary necrosis, the amount of LDH released by NHOst cells was analyzed immediately after heat stress and 24 h post-treatment, and compared to untreated control cells (Figure 5). The LDH assay demonstrated that heat stress at 46°C immediately induced human osteoblasts, which lead to significant release of LDH 24 h after heat stress ($p<0.05$).

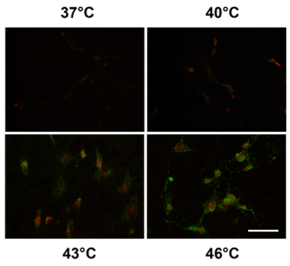


Figure 4: Heat stress induces apoptosis and necrosis in normal human osteoblasts. Annexin-V-fluorescein staining (green) indicates apoptotic cells; propidium iodide staining (red) indicates necrotic cells. Colocalization of Annexin-V-fluorescein and propidium iodide is indicated in yellow. Scale bar = 200 μm.

## 4. Discussion

Hyperthermia at 42-46°C for 30-60 min induces cell death in many cell types (Adachi *et al.*, 2009; Zhao *et al.*, 2006). In this study, we used normal human osteoblast as a model to study the heat stress because it provided more clinical relevant results than animal cells or osteosarcoma cells. This cell also retained the differentiated osteoblast phenotypes and tested positive for the alkaline phosphatase and the formation of mineralization nodules. We found that exposure of normal human osteoblast cells to heat stress from 43°C to 46°C for 30 min resulted in cell death, while no significant effects were observed after exposure to heat stress at 40°C. These results are in contrast to the results of previous studies. For example, in Li *et al.* (1999) reported that exposure to heat shock at 48°C induced both necrosis and apoptosis in rat calvarial osteoblasts; however, apoptotic or necrotic cell death was not observed after heat treatment at

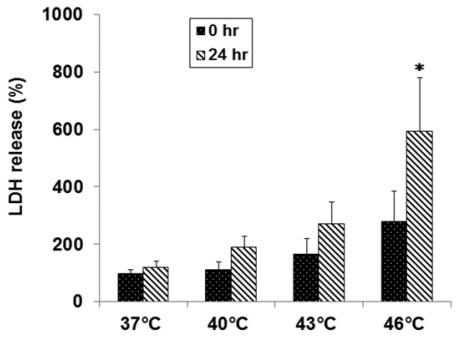


Figure 5: Effect of heat stress on lactate dehydrogenase (LDH) release by normal human osteoblasts. LDH release was expressed as the mean ± SE percentage value of untreated control cells (100%). *, $p<0.05$ vs. untreated control cells.

42°C or 45°C. In another study, hyperthermia at 43°C for 45 min decreased the cell viability of the RPC-C2A dental pulp cell line at 12 h; however, cell viability increased after 24-48 h (Kitamura *et al*., 2005). In contrast, hyperthermia at 42°C for 1 h significantly inhibited the proliferation of the human osteosarcoma cell lines HOS85, MG-63 and SaOS-2 and decreased alkaline phosphatase activity, a marker of osteoblast differentiation (Trieb *et al*., 2007). However, contradictory results were reported in other osteoblast models, demonstrating that heat shock at 41-44°C for 1 h enhanced alkaline phosphatase and mineralized matrix formation of a telomerase-immortalized human mesenchymal stem cell (Norgaard *et al*., 2006). Our results suggest that the heat shock response or thermotolerance may not occur in normal human osteoblasts and that hyperthermia results in irreversible cell damage at sublethal levels of heat stress. A lack of cytoprotection and absence of induction of HSPs has also been reported in normal human dermal fibroblast cells exposed to heat at 42±0.5°C (Jones *et al*., 2003); whereas the induction of HSP proteins was observed in keratinocytes after exposure to 42°C and 47°C (Merwald *et al*., 2006). The varying responses of cells to heat stress may be due to differences in the cellular defense mechanisms of different cell types. In tumor or cancer cells, the expression of many genes including HSPs is usually high and HSPs could be released in a highly stressed microenvironment (Calderwood *et al.*, 2012). However, normal finite cell lines, such as NHOst used in this study, usually express moderate amount of HSP proteins for normal functions and may respond differently to environmental stress.

Many studies have demonstrated that transient, nonlethal hyperthermia can induce cytoprotection and the expression of HSPs in many cell types (Saito *et al*., 2005; Trieb *et al*., 2007). However, in this study, Western blot analyses revealed no differences in the intracellular expression levels of HSP60 protein in normal human osteoblasts and osteoblasts after heat treatment at 40°C, 43°C or 46°C for 5-30 min. HSP60, unlike HSP27 or HSP70, has been reported to have multiple intracellular roles. HSP60 is mostly localized to the mitochondrial matrix and exerts pro-survival functions in some cell types and pro-apoptotic functions in other cell types. In addition, the mechanisms by which apoptosis is induced can also determine whether HSP60 plays a pro-survival or pro-death role. Chandra *et al*. (2007) demonstrated that some apoptotic systems induced mitochondrial release of HSP60 into the cytoplasm while other apoptotic systems increased the expression of HSP60 in the cytosol without apparent mitochondrial release. Growing evidence also supports the pro-apoptotic role of mitochondrial HSP60 and anti-apoptotic role of cytosolic HSP60 (Arya *et al*., 2007). For example, studies in Jurkat cells indicated that formation of HSP60 and procaspase-3 complexes in the mitochondria promote apoptosis (Samali *et al*., 1999). In contrast, hypoxia leads to translocation of HSP60 from the cytosol to the plasma membrane, without changing the total levels of HSP60 in cardiac myocytes (Gupta *et al*., 2002), thus allowing the pro-apoptotic protein BAX to translocate to mitochondria and induce apoptosis (Gupta *et al*., 2005). Recently, translocation of HSP60 to the cell surface has been reported to induce apoptotic membrane blebbing in apoptotic monocytic cells (Goh *et al*., 2011). In contrast, we observed no dramatic changes in the subcellular localization of HSP60 in the mitochondria, cytoplasm or plasma membrane of human osteoblasts after heat stress, which suggests that normal human osteoblasts may lack a heat shock response. The lack of heat shock response may be due to the defect in heat shock transcription factor (HSF)-mediated transactivation of HSP genes in normal human osteoblasts similar to the previous report of some cell lineages (Oommen *et al*., 2012). Additionally, many studies investigating heat stress have observed significant stress responses or the induction of HSPs after exposure of the cells to prolonged 1-2 h heat shock treatments (Diller, 2006; Rylander *et al*., 2005). Therefore, it is also possible that the time course used in this study, which is similar to the time taken for bone surgery in clinical situations, may be too short to induce HSP60 protein expression or HSP60 translocation in human osteoblasts. The findings of this study provide insight into the possible effects of sublethal heat stress, below the previously reported threshold at 47°C for 1 min, which may occur during bone surgery on osteoblast viability. Therefore, careful surgical techniques to avoid excessive heat on the surrounding bones are necessary to prevent osteoblast cell death, thereby improving the treatment outcome.

Several studies have suggested that HSPs are released from dying, necrotic cells (Basu *et al*., 2000; Saito *et al*., 2005) while other studies have shown that viable cells can release HSPs (Lancaster *et al*., 2004; Merendino *et al.*, 2010). For example, HSP60 can be detected in the conditioned medium of HeLa cells 48 h after treatment with 1 mM acrylamide, exposure to 43°C for 1 h followed by a freeze-thaw process, or a freeze-thaw process which mimics necrosis (Basu *et al*., 2000; Saito *et al*., 2005). Our data demonstrated the presence of HSP60 in the conditioned medium of necrotic normal human osteoblast cells after four freeze-thaw cycles; however, we could not detect extracellular HSP60 in the con-

ditioned medium of heat-stressed osteoblasts. The Annexin-V/propidium iodide apoptosis assay and LDH assay demonstrated that apoptosis, necrosis and also possibly secondary necrosis were induced in heat-treated human osteoblasts. Our observations are not inconsistent with previous studies which have shown that necrotic cells can passively release HSPs into the extracellular milieu which may trigger an inflammatory response, while most early apoptotic cells retain their intracellular components to prevent inflammation. However, late apoptosis may be accompanied by mitochondrial degeneration and the release of intracellular components such as HSP60 in some cell types (Beere, 2004). The mechanisms by which HSP60 is released or secreted into the extracellular space are not well understood. It is possible that HSP60 may simply passively leak out of the damaged cell membrane of necrotic human osteoblasts; however, the factors which explain why HSP60 was undetectable in the conditioned medium of heat-stressed cells remain to be elucidated. As the function of extracellular HSP60 has been identified as a proinflammatory signal and apoptotic inducer, and HSP60 can be detected in the plasma of estrogen-deficient rats and women, the roles of extracellular HSP60 as a regulator of bone inflammatory processes after bone damage are of interest and require further investigation.

## 5. Conclusion

Exposure of normal human osteoblasts to heat stress from 43°C to 46°C for as little as 5 min can induced significant cell death. However, necrotic cell death, but not heat-induced cell death, leads to the extracellular release of HSP60 protein by normal human osteoblasts.

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