

Effects of Heat Stress and Starvation on Clonal Odontoblast-like Cells

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Abstract

Introduction: Heat stress during restorative procedures, particularly under severe starvation conditions, can trigger damage to dental pulp. In the present study, we examined effects of heat stress on odontoblastic activity and inflammatory responses in an odontoblast-like cell line (KN-3) under serum-starved conditions. **Methods:** Viability, nuclear structures, and inflammatory responses of KN-3 cells were examined in culture medium containing 10% or 1% serum after exposure to heat stress at 43°C for 45 minutes. Gene expression of extracellular matrices, alkaline phosphatase activity, and detection of extracellular calcium deposition in cells exposed to heat stress were also examined. **Results:** Reduced viability and apoptosis were transiently induced in KN-3 cells during the initial phases after heat stress; thereafter, cells recovered their viability. The cytotoxic effects of heat stress were enhanced under serum-starved conditions. Heat stress also strongly up-regulated expression of heat shock protein 25 as well as transient expression of tumor necrosis factor- α , interleukin-6, and cyclooxygenase-2 in KN-3 cells. In contrast, expression of type-1 collagen, runt-related transcription factor 2, and dentin sialophosphoprotein were not inhibited by heat stress although starvation suppressed ALP activity and delayed progression of calcification. **Conclusions:** Odontoblast-like cells showed thermoresistance with transient inflammatory responses and without loss of calcification activity, and their thermoresistance and calcification activity were influenced by nutritional status. (*J Endod* 2011;37:955–961)

Key Words

Heat stress, inflammatory response, odontoblast-like cells, starvation, thermoresistance

Heat stress as a result of cavity preparation can damage the dental pulp (1). Although studies have shown that transient heat stress induces apoptosis in dental pulp cells, the cells have also been shown to exhibit thermoresistance (2–4). However, heat stress can result in pulp necrosis when extensive procedures are performed (1). It is also known that nutritional status maintained by the microcirculatory system is critically important for cell and tissue homeostasis in dental pulp (5). In restorative procedures, local anesthetic agents containing vasoconstrictors are often administered, and these give rise to decreased pulpal blood flow (6), resulting in hypoxia and starvation conditions in the dental pulp. We previously showed that hypoxia induces cell-cycle arrest and cell death in dental pulp cells and that the viability of pulp cells can be recovered when hypoxia-exposed cells are cultured under normoxic conditions, thus suggesting that dental pulp cells show hypoxia resistance (7).

Odontoblasts or odontoblast-like cells are differentiated from precursor cells or stem cells in dental pulp (8, 9) and are known to play important roles in the defensive responses of dental pulp (10, 11). External stimulation promotes the proliferation of dental pulp stem cells that differentiate into odontoblasts or odontoblast-like cells (12, 13) and form the tertiary dentin (14, 15). Several studies have suggested that odontoblasts participate directly or indirectly in the inflammatory responses of dental pulp through dentinal tubules (16). However, the responses of odontoblasts or odontoblast-like cells to heat stress under starvation conditions are poorly understood. In the present study, we examined the effects of heat stress under fetal calf serum (FCS)-abundant or -starved conditions in a rat clonal odontoblast-like cell line.

Materials and Methods

Cell Cultures and Heat Stress

A rat clonal odontoblast-like cell line (KN-3) (17) was seeded at a density of $9.0 \times 10^3/\text{cm}^2$ in culture dishes or plates and cultured with minimum essential medium Eagle alpha modification (Sigma-Aldrich, St Louis, MO) containing 10% (abundant conditions) or 1% (starved conditions) heat-inactivated FCS (JRH Bioscience, Lenexa, KS), 100 $\mu\text{g}/\text{mL}$ streptomycin (Sigma-Aldrich), and 100 U/mL penicillin (Sigma-Aldrich) in a humidified atmosphere of 5% CO_2 at 37°C. After 24 hours, KN-3 cells were exposed to heat stress at 43°C for 45 minutes as previously described (4).

For rapid heat stress, culture medium was changed to medium preheated at 43°C, and culture dishes or plates were placed on aluminum blocks preheated to 43°C followed by incubation for 45 minutes at 43°C. Thereafter, medium was changed to

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medium preheated to 37°C followed by incubation at 37°C. As a control, non-heat-treated KN-3 cells were cultured in medium containing 10% or 1% FCS at 37°C.

Cell Proliferation Assay

Cell proliferation after exposure to heat stress was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. KN-3 cells were incubated in 96-well plates (AGC Techno Glass, Funabashi, Japan) and exposed to heat stress. At each time point after heat stress, MTS assay was performed using a CellTiter96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Detection of Apoptosis

In order to detect *in situ* DNA strand breaks at the 3'-hydroxyl ends, we performed a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using In Situ Cell Death Detection Kit Fluorescein (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. KN-3 cells cultured on poly-L-lysine-coated coverslips (AGC Techno Glass) for 12, 24, and 36 hours after heat stress were fixed with 4% paraformaldehyde (PFA) (Wako Pure Chemical, Osaka, Japan) in phosphate-buffered saline (PBS) for 60 minutes at room temperature. Cells were incubated in 0.1% Triton-X100 (Wako) in 0.1% sodium citrate (Wako) as permeabilization solution for 2 minutes on ice followed by incubation with TUNEL reaction mixture for 60 minutes at 37°C in the dark. As a positive control, non-heat-treated cells were treated with DNase I (Roche) (20 U/mL), and, as a negative control, non-heat-treated cells were treated with Label Solution (Roche).

For nuclear staining, KN-3 cells cultured on coverslips were fixed with 4% PFA/PBS for 15 minutes, rinsed with PBS, and stained with 0.1 µg/mL of 4'-diamino-2-phenylindole (Dojindo Molecular Technologies, Kumamoto, Japan) for 5 minutes.

Cell-cycle Analysis

KN-3 cells were cultured in medium containing 10% or 1% FCS in 60-mm dishes (AGC Techno Glass). Non-heat-treated or heat-stressed cells were suspended in a hypotonic solution (0.1% Triton X-100, 1 mmol/L Tris/HCl [pH = 8.0], 3.4 mmol/L sodium citrate, and 0.1 mmol/L EDTA) and stained with 5 µg/mL propidium iodide (Dojindo), after which cell-cycle distribution was analyzed using a FACScalibur flow cytometer EPCS XL (Beckman Coulter, Fullerton, CA).

Reverse Transcriptase Polymerase Chain Reaction

KN-3 cells were cultured in 60-mm dishes (AGC Techno Glass). The total RNA was extracted from non-heat-treated or heat-stressed cells using an RNeasy Plus Mini kit (QIAGEN, Hilden, Germany) at each time point after heat stress. To remove genomic DNA contamination, RNA samples were treated with a TURBO DNA-Free Kit (Applied Biosystems, Foster City, CA) for 30 minutes at 37°C. Complementary DNA was synthesized from 1 µg of total RNA using the Transcriptor High Fidelity cDNA Synthesis kit (Roche). Polymerase chain reaction (PCR) amplification was performed with *Taq* polymerase (TaKaRa Ex Taq; TAKARA BIO, Otsu, Japan). Specific primers designed for heat shock protein 25 (HSP25), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), cyclooxygenase-2 (COX2), type-1 collagen (Col-1), runt-related transcription factor 2 (Runx2), and dentin sialophosphoprotein (DSPP) are listed in Table 1. Amplification was performed in a PCR thermal cycler for 22 to 45 cycles as follows: 94°C for 30

seconds, 50° to 68°C for 30 seconds, and 72°C for 30 seconds. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as internal standards. After PCR amplification, products were analyzed by 2% agarose gel (Wako) electrophoresis. Ratios of HSP25, TNF- α , IL-6, COX2, Col-1, Runx2, and DSPP expression to GAPDH expression were analyzed using Image J (National Institutes of Health, Bethesda, MD).

Alkaline Phosphatase Activity

KN-3 cells were incubated in 96-well plates (AGC Techno Glass) and exposed to heat stress. At each time point after heat stress, alkaline phosphatase (ALP) activity was measured using *p*-nitrophenylphosphate assay (LabAssay ALP Kit, Wako). After 15 minutes of incubation at 37°C, absorbance of *p*-nitrophenylphosphate at 405 nm was determined using a microplate reader, and the specific activity of ALP (µg/protein per 30 minutes) was calculated. Protein contents were measured with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Detection of Extracellular Calcium Deposition

Mineralized extracellular matrix was stained using the von Kossa staining technique. KN-3 cells were incubated in culture medium containing 10% or 1% FCS in 35-mm dishes (AGC Techno Glass) in a humidified atmosphere of 5% CO₂ at 37°C for 24 hours and exposed to heat stress. After 7 days, culture medium was changed to osteogenic differentiation medium consisting of minimum essential medium Eagle alpha modification containing 10% FCS with 50 µg/mL ascorbic acid (Sigma-Aldrich), 10 mmol/L β -glycerophosphate (Sigma-Aldrich), 100 µg/mL streptomycin, and 100 U/mL penicillin followed by culture in a humidified atmosphere of 5% CO₂ at 37°C. At 2 and 4 weeks of culture, specimens were fixed with 3.7% PFA/PBS for 15 minutes and incubated in 0.5% silver nitrate (Wako) for 1 hour under light conditions followed by incubation in 0.3% sodium thiosulfate pentahydrate (Wako) for 5 minutes. Five samples stained with the von Kossa technique were used for semiquantitative analyses. The five fields, one central and four surrounding fields on each dishes, were selected randomly, and the percentage of calcified nodule areas formed on the dishes was calculated using Image J (National Institutes of Health). This analysis was conducted as a randomized double-blind study.

Statistical Analysis

Statistically significant differences in the MTS assay ($n = 6$), reverse transcriptase PCR assay ($n = 3$), ALP activity ($n = 3$), and the percentage of calcified nodules ($n = 5$) among the four groups (non-heat-treated cells and heat-stressed cells cultured in medium containing 10% or 1% FCS) were determined using the Student *t* test. All data are expressed as means \pm standard deviation.

Results

Thermoresistance and Apoptosis of KN-3 Cells after Heat Stress

The effects of heat stress on the proliferation of KN-3 cells are shown in Figure 1A. Throughout the culture period, the viability of KN-3 cells increased in control (non-heat-treated) groups. In contrast, the viability of heat-stressed cells was transiently decreased at 12 hours under both FCS-abundant and -starved conditions. After the transient reduction in cell viability, the viability of heat-stressed cells recovered, and the proliferation rate was significantly higher in 10% FCS medium than in 1% FCS medium. The expression of HSP25 after heat stress is

TABLE 1. Sequences of Primer Pairs Used for Reverse Transcription PCR

Gene	Sequence	Annealing temperature	Cycle	Amplicon (bp)	References
HSP25	Forward 5'-GTAAAGACCAAGGAAGGCCTGG-3' Reverse 5'-CTACTTGGCTCCAGACTGTTCC-3'	68°C	22	279	18
Col-1	Forward 5'-CCAATCTGGTCCCTCCAC-3' Reverse 5'-TGGTAAGGTTGAATGCACTT-3'	62°C	35	214	19
Runx2	Forward 5'-CCAGATGGGACTGTGGTTAC-3' Reverse 5'-ACTTGGTGCAGAGTTCAGGG-3'	58°C	38	381	17
DSPP	Forward 5'-CACATCCAGGAACCGCAGCAC-3' Reverse 5'-CCTTACTCTCCTTGCCTC-3'	50°C	45	330	19
TNF- α	Forward 5'-CGTCGTAGCAAACCACCAAGC-3' Reverse 5'-ACCAGGGCTTGAGTCAGTC-3'	64°C	38	296	20
IL-6	Forward 5'-CTTCCAGCCAGTTGCCCTTCT-3' Reverse 5'-GAGAGCATTGGAAGTTGGGG-3'	63°C	27	496	20
COX2	Forward 5'-AATGAGTACCGCAAACGCTT-3' Reverse 5'-ATCTAGTCTGGAGTGGGAGG-3'	68°C	28	420	21
GAPDH	Forward 5'-ACCACAGTCCATGCCATCAC-3' Reverse 5'-TCCACCACCTGTTGCTGTA-3'	62°C	23	452	19

shown in Figure 1B. Just before the recovery of cell proliferation shown in Figure 1A, intense expression of HSP25 at 3, 6, and 12 hours after heat stress was observed in both 10% and 1% FCS medium.

We also observed apoptosis of KN-3 cells attached to cover slips on the TUNEL assay after heat stress (Fig. 1C). Without heat stress, TUNEL-positive signals were slightly detectable in the cells cultured with 1% FCS medium but not in 10% FCS medium. At 24 hours after exposure to heat stress, TUNEL-positive signals were clearly observed in both 10% and 1% FCS medium. The morphological changes in KN-3 cells in 10% FCS medium after heat stress are shown in Figure 1D. At 12 hours after heat stress, some cells appeared round and became detached from the bottom of the dishes (data not shown), and the KN-3 cells attached to the bottom of dishes showed two different features: apoptotic cells with the typical nuclear fragmentation and surviving cells with normal nuclei. At 36 hours after heat stress, a phagocytotic-like phenomenon was observed, with scavenger-like KN-3 cells apparently phagocytosing apoptotic cells. A similar state was observed in 1% FCS medium after heat stress (data not shown).

The effects of heat stress and starvation on the cell-cycle progression of KN-3 cells were also analyzed (Fig. 1E). In non-heat-treated cells, starved conditions (1% FCS medium) increased the number of KN-3 cells in the G1 phase (72.9%), with a concomitant reduction of those in the S (7.7%) and G2/M phases (10.4%), as compared with control cells in 10%-FCS medium (G1, 60.1%; S, 17.6%; and G2/M, 18.2%). Exposure to heat stress markedly increased the cell number of the cells in the G2/M phase (34.5% in 10% FCS and 21.9% in 1% FCS) and the population in the sub-G1 (7.9% in 10% FCS and 12.2% in 1% FCS) and reduced the number of cells in the G1 (52.9% in 10% FCS and 62.1% in 1% FCS) and S phases (4.9% in 10% FCS and 4.0% in 1% FCS) at 12 hours after heat stress. At 24 hours after heat stress, cell number in the G1 (58.4%) and G2/M (17.1%) phases almost returned to preheated levels in 10% FCS medium, but no such return was seen in 1% FCS medium (G1, 47.4%; G2/M, 19.0%).

Inflammatory Response of KN-3 Cells against Heat Stress

In order to examine the inflammatory responses of KN-3 cells against heat stress, the expression of TNF- α , IL-6, and COX2 were analyzed by reverse transcriptase PCR (Fig. 2A and B). These inflammatory molecules were transiently expressed in heat-stressed cells but not in non-heat-treated cells. TNF- α and IL-6 expression peaked at 3 hours after heat stress in 10% FCS medium but peaked at 6 hours after heat stress in 1% FCS medium. Although significant expression

of COX2 messenger RNA was observed at 3, 6, and 12 hours after heat stress in both 10% and 1% FCS medium, significant COX2 expression was detected at 1 day after heat stress only in 1% FCS medium.

Calcification Activity of KN-3 Cells after Heat Stress

In order to evaluate whether heat stress and starvation influences the odontoblastic properties of KN-3 cells, we examined the expression of Col-1, Runx2, and DSPP by reverse transcriptase PCR (Fig. 3A). There were no significant differences in the amounts of these messenger RNAs between non-heat-treated and heat-stressed cells in both 10% and 1% FCS medium.

The ALP activity of KN-3 cells after heat stress is shown in Figure 3B. The ALP activity was higher in cells cultured in 10% FCS medium when compared with those in 1% FCS medium and was elevated throughout the 7-day culture period. Significant differences in ALP activity were observed between non-heat-treated and heat-stressed cells in 1% FCS medium at 0.5, 1, 3, and 7 days into the culture period.

We also examined the effects of heat stress and starvation on the formation of calcified nodules by KN-3 cells (Fig. 3C and D). Non-heat-treated and heat-stressed cells were cultured in medium containing 10% or 1% FCS for 1 week, and medium was subsequently exchanged with osteogenic differentiation medium followed by culture for 2 or 4 weeks. Calcified nodules in all groups were formed in a time-dependent manner. There were no differences in the formation of calcified nodules between non-heat-treated and heat-stressed cells, whereas the formation of calcified nodules by cells cultured in 1% FCS medium for the first week was significantly lower when compared with cells cultured in 10% FCS medium for the first week.

Discussion

Heat is known to be the most severe stress on dental pulp during restorative procedures (22). It was previously shown that increases in pulpal temperature of more than 5°C from physiological conditions can damage dental pulp (23). In the present study, heat stress induced the transient reduction of KN-3 cell viability and G1 arrest as well as a decrease in the cell population in the S phase. Heat stress also induced apoptosis in KN-3 cells, which was confirmed based on the population of sub-G1 cells and the detection of TUNEL-positive cells, whereas some cells survived and proliferated after heat stress, as previously shown in clonal dental pulp cells exposed to heat stress (4). Furthermore, the expression of HSP25, a rodent homolog of human HSP27 (24), was strongly up-regulated in heat-stressed KN-3 cells before the proliferation of surviving cells. It is known that cell-cycle arrest is essential for

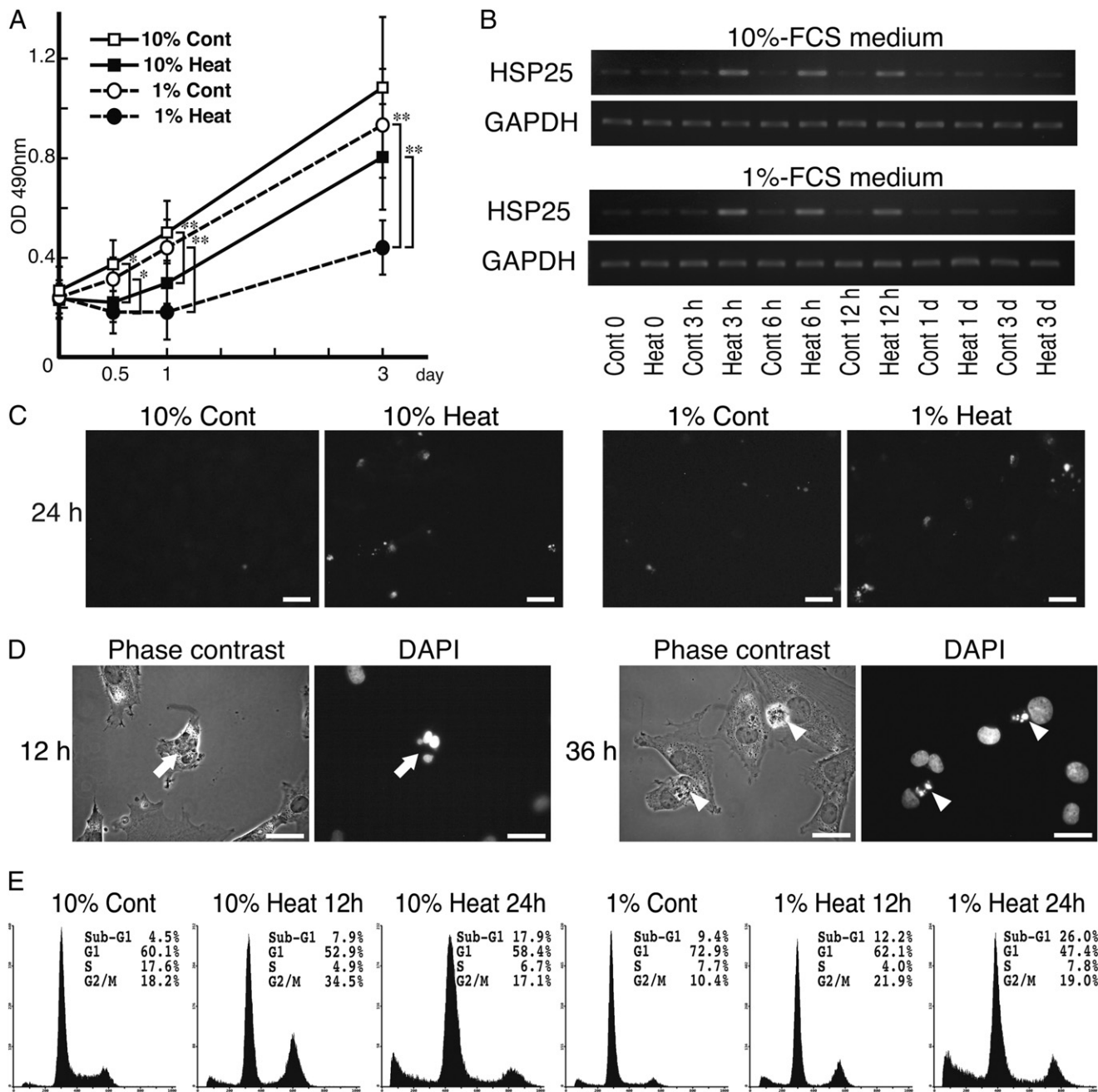


Figure 1. (A) MTS assay for KN-3 cell proliferation after heat stress under FCS-abundant or -starved conditions. The number of heat-stressed cells cultured in 10% and 1% FCS medium decreased after 12 hours and thereafter increased. The proliferation capacity of KN-3 cells cultured with 10% FCS medium was significantly higher when compared with those cultured with 1% FCS medium at 3 days after heat stress. Data are expressed as means \pm standard deviation ($n = 6$, $**P < .01$, $*P < .05$). (B) Reverse-transcriptase PCR analysis for the expression of HSP25 in non-heat-treated or heat-stressed KN-3 cells cultured under FCS-abundant or -starved conditions. Intense expression of HSP25 was observed in cells cultured in 10% and 1% FCS medium at 3, 6, and 12 hours after heat stress. (C) TUNEL assay of nontreated and heat-stressed KN-3 cells. Some TUNEL-positive signals were observed in non-heat-treated cells in 1% FCS medium, and numerous positive signals were observed in heat-stressed cells cultured with 10% and 1% FCS medium. Few signals were noted in non-heat-treated cells cultured with 10% FCS medium. Scale bars indicate 50 μm . (D) Nuclear morphologies of KN-3 cells after heat stress after 12 and 36 hours. Apoptotic cells, showing nuclear fragmentation and apoptotic corpuscles, were detected at 12 hours (arrow) and 36 hours (arrowhead). At 36 hours, scavenger-like cells were also observed adjacent to the apoptotic cells. Scale bars indicate 30 μm . (E) Representative results of cell-cycle distribution of the non-heat-treated and heat-stressed KN-3 cells under FCS-abundant or -starved conditions. Serum-starved conditions increased the number of KN-3 cells in the G1 phase and the population of cells in the sub-G1, with concomitant reductions of those in the S and G2/M phases. Exposure to heat stress markedly increased cell number in the G2/M phase and the population in the sub-G1 at 12 hours after heat stress and reduced the cell number in the G1 and S phases in 10% and 1% FCS medium at 24 hours.

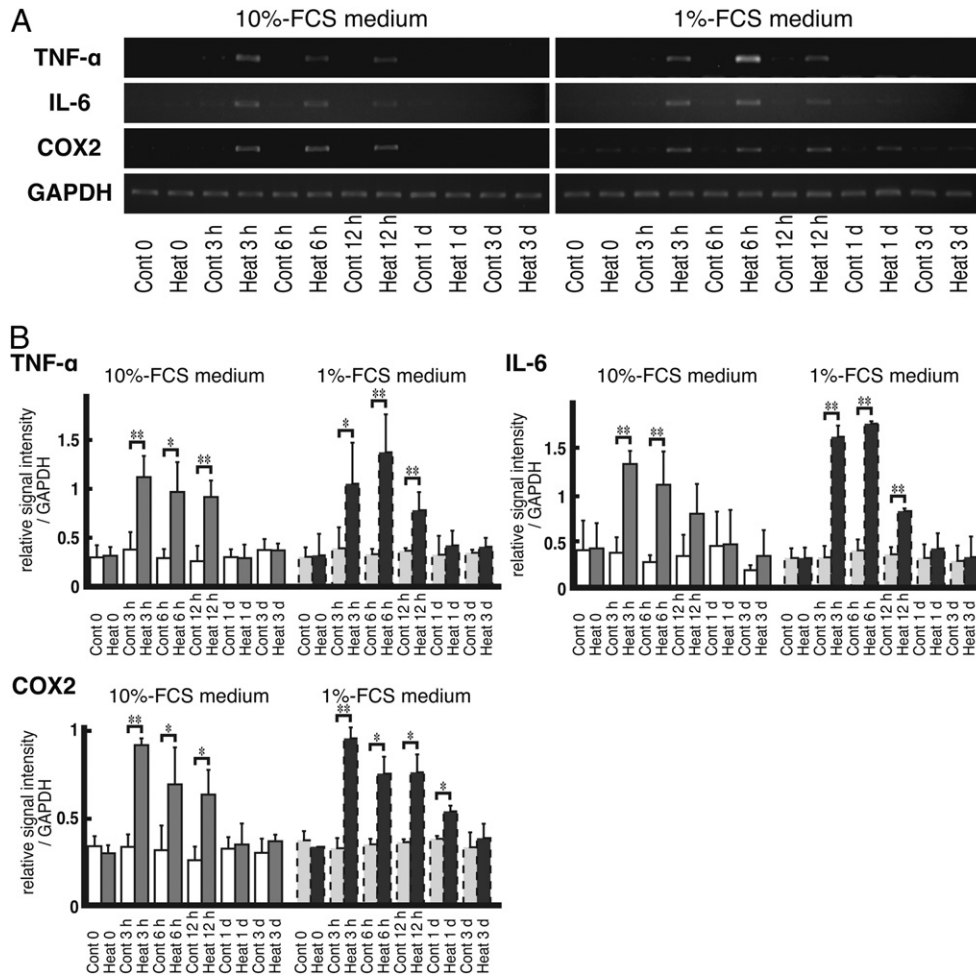


Figure 2. (A) Reverse-transcription PCR analysis for expression of TNF- α , IL-6, and COX2 in non-heat-treated or heat-stressed KN-3 cells cultured under FCS-abundant or -starved conditions. TNF- α , IL-6, and COX2 messenger RNAs were transiently expressed in KN-3 cells after heat stress. (B) Ratios of TNF- α , IL-6, and COX2 expression versus GAPDH expression. TNF- α and IL-6 expression peaked at 3 hours after heat stress in 10% FCS medium but peaked at 6 hours after heat stress in 1% FCS medium. Significant expression of COX2 messenger RNA was observed at 3, 6, and 12 hours after heat stress in 10% and 1% FCS medium. Furthermore, significant COX2 expression was detected at 1 day after heat stress only in 1% FCS medium. Data are expressed as means \pm standard deviation ($n = 3$, ** $P < .01$, * $P < .05$).

the maintenance of viability under environmental conditions that inhibit normal regulation of cell growth (25) and that HSPs play critical roles in protection from the cellular damage associated with various stress stimuli (26, 27). These results suggest that odontoblast-like KN-3 cells have the ability to resist heat stress through regulation of the cell cycle and the induction of HSP25. In the comparison of nutritional conditions, reductions in the population of cells in the S phase, G1 arrest, and cell death induced by heat stress were all seen at higher levels in cells cultured with 1% FCS medium. The proliferation rate of surviving cells cultured with 10% FCS medium was also higher than that of cells in 1% FCS medium after heat stress. These results suggest that starvation enhances the cytotoxic effects of heat stress.

Next, the effects of heat stress and nutritional conditions on inflammatory responses of KN-3 cells were examined. It is known that TNF- α , IL-6, and COX2 are inducible inflammatory mediators and the messenger RNA expression of TNF- α , IL-6, and COX2 corresponds to the onset of pulpitis (28). It was previously shown that bacterial lipopolysaccharide (LPS), which is one of factors related to onset of pulpitis, induces the expression of these messenger RNAs in KN-3 cells (29). In the present study, expression of TNF- α , IL-6, and COX2 were tran-

siently induced in the initial phases after heat stress, thus suggesting that KN-3 cells show immediate inflammatory responses against heat stress similar to LPS. When KN-3 cells were cultured under starved conditions, the peak and/or disappearance of expression of these inducible inflammatory mediators were delayed. Thus, nutritional status may also influence the inflammatory responses of KN-3 cells against heat stress.

The effects of heat stress and nutritional conditions on calcification activity by KN-3 cells were also analyzed. The expression of Col-1, Runx2, and DSPP in KN-3 cells was not influenced by heat stress under either nutritional state. The reduction of ALP activity by heat stress was only seen in 1% FCS medium and the formation of calcified nodules by the cells precultured in 1% FCS medium for the first week was clearly delayed, whereas heat stress had no effect on the formation of calcified nodules. It is generally accepted that ALP activity and the expression of Col-1, DSPP, and Runx2 are markers of odontoblast differentiation and odontoblastic function (30–35). The present results indicate that the effects of nutritional state are substantial on the differentiation and calcification of odontoblast-like cells, whereas the effects of heat stress are mild. It was previously shown that LPS suppresses ALP activity, the

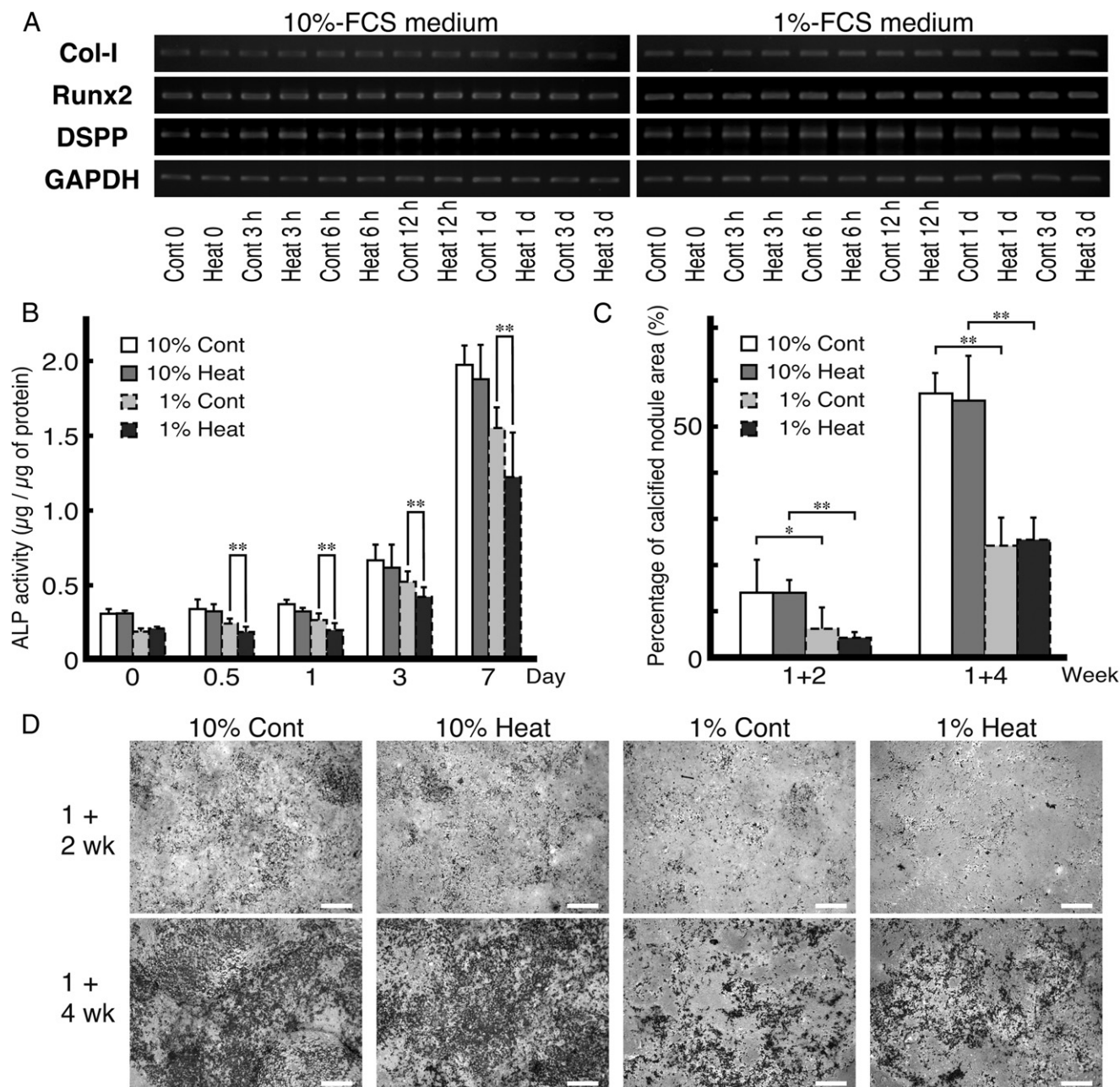


Figure 3. (A) The expression of Col-1, Runx2, and DSPP in non-heat-treated and heat-stressed KN-3 cells cultured under FCS-abundant or -starved conditions. No significant differences between non-heat-treated and heat-stressed cells were observed when cultured under FCS-abundant or -starved conditions. (B) ALP activity of non-heat-treated and heat-stressed KN-3 cells under FCS-abundant or -starved conditions. Significant differences were observed between non-heat-treated and heat-stressed cells in 1% FCS medium at 0.5, 1, 3, and 7 days. Data are expressed as means \pm standard deviation ($n = 3$, $**P < .01$). (C) The percent area of calcified nodules produced by non-heat-treated and heat-stressed KN-3 cells precultured in 10% or 1% FCS medium. There were no significant differences in the formation of calcified nodules between non-heat-treated and heat-stressed cells in 10% and 1% FCS medium. However, calcified nodule levels after preculture in 1% FCS medium for 1 week were significantly lower when compared with 10% FCS medium independent of heat stress. Data are expressed as means \pm standard deviation ($n = 5$, $**P < .01$, $*P < .05$). (D) Photomicrographs of calcified nodules by non-heat-treated and heat-stressed KN-3 cells precultured in 10% or 1% FCS medium. Scale bars indicate 500 μm .

expression of Runx2 and DSPP, and the formation of calcification nodules (17). Physical stresses, such as heat stress, may not have an effect on the odontoblastic properties of odontoblast-like cells, which is in contrast to the effects of biochemical stresses, such as LPS and starvation.

Taken together, the present study indicates that heat stress induces cytotoxic effects, such as apoptosis, on odontoblast-like cells, but some

cells survive, exhibiting thermoresistance with inflammatory responses to heat stress. The cytotoxic effects of heat stress were enhanced under starved conditions, but heat stress had little effect on differentiation and calcification activity of odontoblast-like cells. Our results suggest that odontoblast-like cells are able to survive and maintain their functional properties during pulp wound healing after proper clinical dental procedures.

Acknowledgments

The authors deny any conflicts of interest related to this study.

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