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THE EFFECTS OF HYPO- AND HYPERTHERMIA ON SPARC IN NORMAL HUMAN OSTEOBLAST CELLS

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ABSTRACT

SPARC (secreted protein acidic and rich in cysteine) is a matricellular protein involved in cell proliferation, adhesion, movement and it is associated with osteogenesis by balancing bone formation and resorption. *In-vitro* experiments have shown that SPARC is responsible for assembly and organizing cell extracellular matrix (ECM) while a chaperone-like role has been shown to protect proteins during thermal stress conditioning. New treatment approaches are interested to explore bone regenerative potential through temperature stress conditioning. Therefore, the aim of this study was to look at the expression of SPARC protein and mRNA in Normal Human Osteoblast (NHOst) cells after short and prolonged exposure to hypo- and hyperthermia condition.

NHOst cells were exposed to hypothermia (27°C), hyperthermia (39°C), and control (37°C) for 12h, 24h, 72h and 5 days. NHOst cell proliferation rate was measured using MTS-based assay. SPARC protein and mRNA was determined by Confocal Laser Scanning Microscope (CLSM) and Real-Time PCR respectively.

Prolonged temperature stress conditioning influenced both transcriptional and translational levels of SPARC. SPARC expression during long term exposure to hypo- and hyperthermia followed a bell-shaped response curve with a peak at 24h for both protein and mRNA. However, prolonged stress significantly reduced SPARC protein expression for hypo- and hyperthermia and mRNA expression for hyperthermia. We demonstrated that levels of SPARC protein expression responded in a time- and stress severity-dependent manner. Interestingly, exposure to prolonged hypothermia significantly up-regulated levels of SPARC mRNA after 24h up to day 5 although cell proliferation rate was significantly decreased.

Expression pattern for SPARC are distinct different to cell viability, suggesting that SPARC has no influence on cell viability during prolonged exposure. Regulation of SPARC mRNA under prolonged hypothermia suggests that SPARC may be involved in cold shock response.

1.0 Introduction

Gradual decrease in number of bone forming osteoblast cells and activity contributes to brittle bone diseases such as osteoporosis, osteopenia and osteogenesis imperfecta [1] by shifting the balance in bone remodeling towards resorption. SPARC/osteonectin is the most abundant matricellular protein in bone and is associated with balancing bone formation and resorption. Evidently shown, SPARC-null (-/-) mice developed low-turnover osteopenia [2] with reduced bone mass. In contrast to the standard treatment methods for brittle bone diseases, new treatment approaches are interested to

explore bone regenerative potential through temperature stress conditioning.

Although few studies have explored thermal stress conditioning for bone regeneration [3], mechanical stress is the most investigated stress conditioning method by far [4]. Numerous studies on bone [5], cartilage [6], teeth [7], and skin [8] demonstrated that acute severe hyperthermia (40-42°C) increased cellular metabolism and induced tissue regeneration without being cytotoxic to the cells [5, 6]. Acute incubation of osteoblast cells for 1h at 39-41°C using heating devices increased cell proliferation rate and bone alkaline phosphatase *in vitro* [5]. Meanwhile, thermally-treated osteoblast cells enhanced secretion of bone markers (osteocalcin, osteopontin and SPARC) and ability of bone cells to mineralize [7].

Generally mammalian cells respond to temperature stress by increasing chaperone protein activity to encourage correct protein folding and prevent protein misfolding [8]. A set of cold (RNA-binding motif 3; Rbm3 & Cold-inducible RNA binding protein; CRIP) [9] and heat shock proteins (Hsp27, Hsp70 & Hsp47) [10] are synthesized to facilitate proper protein folding [3] under cellular stress. SPARC responsible for assembly and organizing cell extracellular matrix (ECM) has recently been shown to function as a chaperone-like protein during heat stress [11] similarly to heat shock proteins. During low temperature stress conditioning (23°C) cells maintained refolding of proteins by secreting SPARC protein [2]. This may explain our results obtained during the Malaysian space flight program, which showed increased SPARC mRNA expression in the temperature adapted ground controls versus 37°C controls (unpublished data). However, expression of SPARC mRNA in brain vessels after 6h and 24h at 32°C of post-ischemia was reduced [12].

Recent studies on chicken embryo chondrocytes and human keratinocytes identified SPARC as a 43-45kDa protein being constantly secreted in large amounts with response to thermal stress and other physiological stressors [3]. However, limited knowledge exists on SPARC expression and chaperone-like role [11] during temperature stress conditioning in osteoblasts cells.

Therefore, this study investigated the effects of prolonged hypo- and hyperthermia on Normal Human Osteoblast (NHOst) cells with respect to SPARC protein and mRNA expression with response to temperature conditioning.

2.0 Materials and Methods

2.1 Cell Culture

Normal Human Osteoblast (NHOst) cells were purchased from LONZA, USA. Cells were cultured in T-25 Flasks (Orange Scientific, Belgium) in Osteoblast Basal Medium (OBM™) supplemented with 10% foetal bovine serum (FBS), 0.1% ascorbic acid, 0.1% gentamicin and 0.1% amphotericin-B. The cells were grown in a water-jacketed incubator at 37°C with 5% CO_2. Confluent NHOst monolayer was split with Accutase every 5 to 6 days and cells at passage 8 were used for all experiments.

2.2 Temperature Treatment

A total of 2.5 x 10^5 cells/cm^2 were seeded per T-25 flask and preincubated at 37°C for 24h. Cells were then exposed to hypothermia (27°C) and hyperthermia (39°C) and control (37°C) for 12h, 24h, 72h and 5 days in a water-jacketed incubator with 5% CO_2. Each temperature experiments was carried out in triplicates (n = 3).

2.3 SPARC Immunofluorescence Staining

A total of 1.0 x 10^4 NHOst cells were cultured on Lab-Tek II chamber slides. Cells were washed with Phosphate Buffered Saline (PBS) and fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature after each temperature interval. SPARC subcellular protein was conjugated with goat anti-mouse IgG-FITC. Slides were mounted with Prolong® Gold Antifade. Slides were stored at 4ºC in the dark until scanned with a Confocal Laser Scanning Microscope (Leica TCS SP5). A total of 50 cells (n = 50) were analysed and fluorescence intensity of SPARC localization in the cell cytoplasm was measured using Leica QWin software.

2.4 Cell Proliferation Assay

NHOst cell proliferation was determined using the MTS-based assay, CellTiter 96® Aqueous One solution. Total of 2.5 x 10^4 cells per well were seeded in 96 well plates. A volume of 20µl CellTiter 96® Aqueous One Solution was added to OBM™ medium contained in each well. Plates were incubated at 37°C for 2h in a humidified 5% CO_2 incubator. Formation of formazan was measured by absorbance at 490nm.

2.5 SPARC Gene Expression

Extraction of total RNA from hypo- and hyperthermia conditioning was done using RNeasy® Mini kit. Total RNA was isolated according to the manufacturer's protocol while the concentration and purity (A_{260}/A_{280}) of eluted RNA was determined using Nanodrop spectrophotometer.

cDNA synthesis was performed using 40ng of total RNA in a 20µl reaction with Sensiscript RT and Oligo-dT primers. Expression of SPARC was analysed using Real-Time PCR.

A cocktail of 1x iQ™ SYBR Green Supermix, 25µM forward (5'-AGA GGA AAC CGA AGA GGA GG-3') and reverse (5'-GGC AAA GAA GTG GCA GGA AG-3') primers, and 2µl cDNA template in a 25µl volume was amplified using iQ™ 5 Real-Time PCR detection system. Normalization of gene expression was done with GAPDH as a housekeeping gene.

2.6 Statistical Analysis

Statistical analysis was performed using the statistical package SPSS (version 17.0) software employing Independent-samples T test to determine the level of significance between control and temperature groups. Statistical significant level was set at $*P < 0.05$, $** P < 0.01$, and $*** P < 0.0001$. All results were calculated and presented as mean ± standard deviation (SD).

3.0 Results and Conclusions

SPARC has been shown to be a stress- induced non-structural, matrix protein in bone with chaperone-like activities [2, 3, 11]. This study demonstrated that exposure to prolonged temperature stress conditioning influenced both transcriptional and translational levels of SPARC in NHOst cells. Severity and duration of temperature stress can induce changes in cell morphology however, no noticeable changes in NHOst morphology were observed after 5 days.

The semi-quantitative immunofluorescence staining method [13] showed distinct changes in fluorescence intensity and subcellular location of FITC-labeled SPARC after hypo- and hyperthermia stress (Fig. 1). SPARC was found widely distributed in the cytoplasm with a higher immunoreactivity of proteins around the nucleus except for thermally stressed cells on day 5 (Fig. 1C) compared to control. The expression of SPARC protein fluorescence intensity profile over 5 days of hypo- and hyperthermia followed a bell-shape curve with a peak at 24h by 104% ($P < 0.05$) and 114.7% ($P < 0.01$) respectively.

Confocal microscopic analysis (Fig. 1) also showed that SPARC protein was present in NHOst nucleus (Fig. 2) for both treated and non-treated NHOst cells except for 5 days of hyperthermia stress. Likewise, Gooden et al., 1999 demonstrated that localization of SPARC protein in nuclear matrix was found in chicken embryonic and bovine aortic endothelial cell [13]. They also could detect the expression of SPARC protein in the interphase nucleus of chicken embryo cells. The group concluded that the concentration of SPARC protein influences DNA synthesis and levels of mRNA [13].

We demonstrated that levels of SPARC protein expression responded in a time- and stress condition-dependent manner.

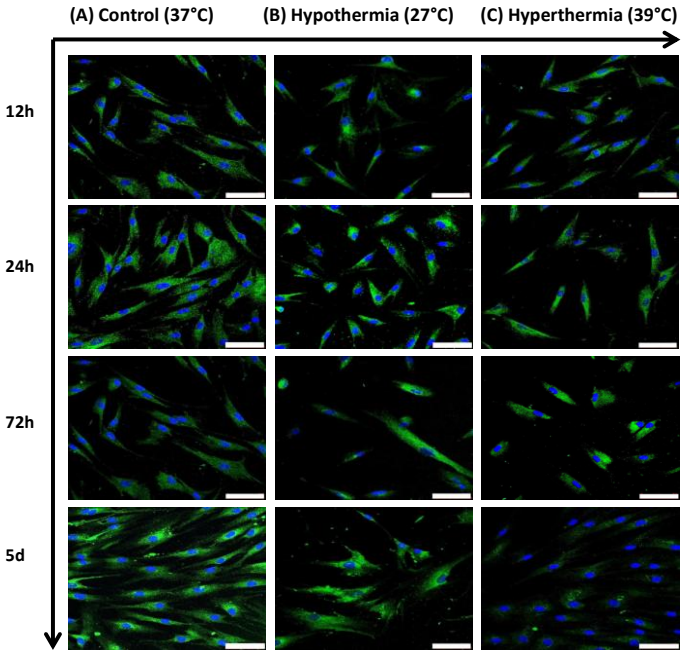

Fig. 1 NHOst cells were fixed and subcellular localization of SPARC protein was conjugated with goat anti-mouse IgG-FITC (green) and DAPI; nucleus (blue) for (A) control, (B) hypothermia and (C) hyperthermia treated cells after 12h, 24h, 72h and 5 d. Cells were viewed with Confocal Laser Scanning Microscope (CLSM) under 40x magnification (Scale bar: 75 µm).

Literature showed that short term exposure to thermal stress accelerates bone osteogenesis by constitutively inducing bone markers (SPARC, OCN & OPN) for bone formation and mineralization in mouse osteoblastic cell line (MC3T3-E1) [15]. Similarly, our short term exposure to temperature stress (24h) increased both protein (Fig. 3) and mRNA expression level. NHOst cells were metabolically active and remained proliferative at 24h under thermal stress condition (Fig. 4). However, prolonged exposure to temperature stress significantly reduced SPARC protein expression for hypo- and hyperthermia and mRNA expression for hyperthermia.

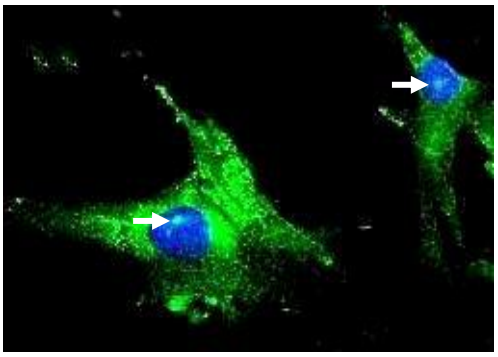

Fig. 2 Localization of SPARC protein (arrow) in NHOst cell nuclear matrix. Cells were viewed with Confocal Laser Scanning Microscope (CLSM) under 40 x magnifications.

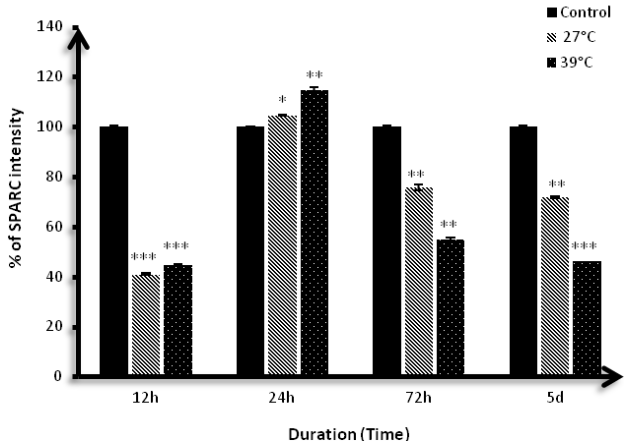

Fig. 3 Localization of SPARC protein in NHOst cell cytoplasm after hypo- and hyperthermia treatment. FITC-labeled SPARC fluorescence was measured through pixel intensity using Leica QWin software. Data obtained from 50 cells (n = 50) is shown as the mean value ± SD of percentage change relative to control (100%). Level of significance between control and temperature was set at *P < 0.05, ** P < 0.01, and *** P < 0.0001 using Independent-samples T test (SPSS).

Interestingly, exposure to hypothermia significantly up-regulated levels of SPARC mRNA after 24h up to day 5 although cell proliferation rate was significantly decreased (Fig. 5).

Our unpublished data demonstrated cold-shock chaperone protein (Rbm3) mRNA was significantly up-regulated under hypothermic conditions to stabilise mRNA while promoting growth arrest to prevent further damage to the cells. Similarly, SPARC showed the same chaperone-like response towards hypothermia by significantly up-regulating SPARC mRNA with reduced cell proliferation rate.

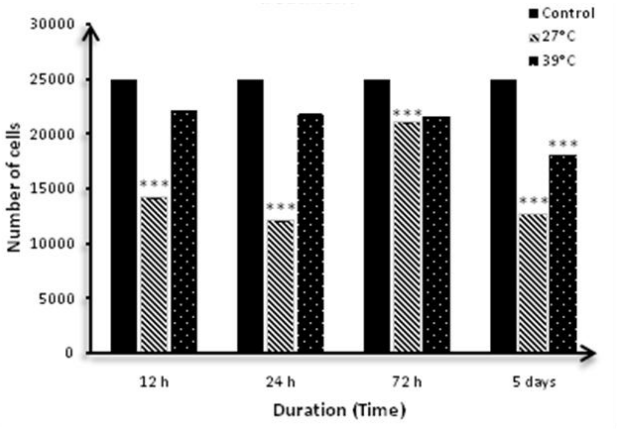

Fig. 4 Effects of hypothermia and hyperthermia on NHOst cell proliferation by MTS assay. NHOst cells treated with moderate and severe hypo- and hyperthermia at 12h, 24h, 72h and 5 days were incubated with MTS-tetrazolium compound at 37°C for 2h. Production of formazan was measured at an absorbance of 490nm. Results represent the means ± SD of six replicate samples (n = 6). ***: P < 0.0001.

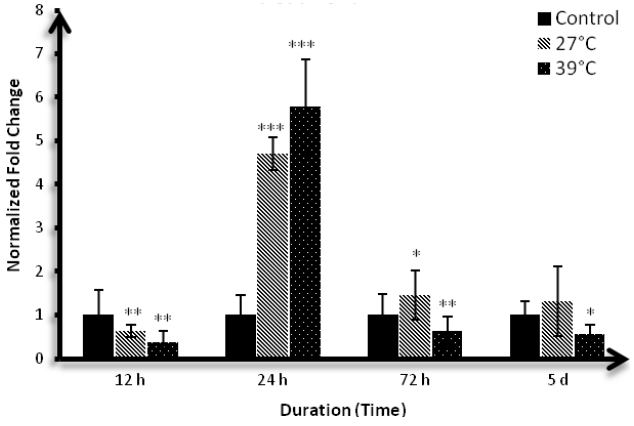

Fig. 5 Real-Time PCR analysis of SPARC mRNA expression in NHOst cells induced by hypothermia and hyperthermia. Normalization of gene expression was done with GAPDH as a housekeeping gene. Level of significance between control and temperature was set at *P < 0.05, ** P < 0.01, and *** P < 0.0001(n = 3) using Independent-samples T test (SPSS).

Our expression levels of SPARC mRNA and protein showed a similar bell-shape like curve trend in thermally challenged NHOst cells up to day 5, confirming that SPARC is a stress induced protein. Neri et al., 1992 demonstrated that acute exposure to severe heat stress at 45.5°C for 10h increased SPARC mRNA expression in chick embryo chondrocytes [3]. We speculate that the expression of SPARC mRNA responses to the degree of stress severity as our acute exposure to mild

thermal stress at 39°C for 12h significantly down-regulated SPARC mRNA. Our results are in agreement with studies done by Sauk et al., 1991 on dog osteoligament cells exposed to prolonged heat stress expressing lower SPARC protein and mRNA compared to control [16].

This study showed that SPARC expression during long term exposure to hypo- and hyperthermia follows a bell-shaped response curve with a peak at 24h. Previous studies have mainly investigated short term exposure. Here we can show that SPARC expression is down-regulated during prolonged exposure with exception of 24h. Why this is the case and what is the function of SPARC at that time point requires further investigations. Additionally expression pattern for SPARC are distinct different to cell viability data, suggesting that SPARC has no influence on cell viability during prolonged exposure. However we observed similar expression pattern of Rbm3 (unpublished data) and SPARC, suggesting that SPARC may be involved in cold shock response.

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Abbreviations

NHOst : Normal Human Osteoblast cells
SPARC : Secreted protein acidic and rich in cysteine
ECM : Extracellular matrix
OCN : Osteocalcin
OPN : Osteopontin
CLSM : Confocal Laser Scanning Microscope

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