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Effect of Long-time Mild Heat Stress on Proliferative, Differentiation and Bone Regeneration Capabilities of Dental Pulp Stem Cells

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Introduction: The application of various strategies, including heat stress, has been attempted to maintain and improve mesenchymal stem cells (MSCs) plasticity and efficiency for bone regeneration. Although cell responses to heat stress are one of the most examined cellular stress responses, most studies, in this context, investigated the effect of that for a short period. Hence, the current study aimed to investigate the properties of MSCs, derived from dental pulp stem cells (DPSCs) following long-term heat stress. **Materials and Methods:** DPSCs was loaded on β-tricalcium phosphate scaffold blocks and cultivated at elevated temperature up to 39 ° C. *In vitro*, DPSC properties, including proliferation, osteogenic differentiation, and bone regeneration *in vivo*, have been evaluated. **Results**: The results of *in vitro* study showed that mild heat stress for a lengthy period could maintain and improve both the proliferative and differentiation potential of DPSCs during *in vitro* expansion and differentiation. Also, bone regeneration *in vivo* showed increased bone regeneration in the cells cultivated at a higher temperature. **Conclusion:** This study provided evidence for the beneficial effects of the administration of mild heat stress for an extended period to maintain and improve DPSC properties, which may serve as a starting point for developing clinically compliant procedures for MSC treatment before transplantation.

Keywords: Bone regeneration; Dental pulp stem cells; Heat stress

Introduction

The application of mesenchymal stem cells (MSCs) in human regenerative medicine is expanding rapidly (1, 2, 3, 4, 5). However, the quantity of MSCs in adult tissues is limited, and many promising tissue engineering approaches require cell expansion, which has been shown to dramatically reduce stem cell plasticity (6, 7, 8). In addition, various studies have shown that a large number of cells die within the early days of transplantation (9, 10, 11). Therefore, to improve the efficiency of stem cell application, the administration of appropriate strategies to enhance the qualitative and quantitative extent of differentiation seems pivotal.

It is believed that stem cells in the adult tissues, under normal physiological conditions, are in the quiescent state (12, 13), a self-care mechanism for preventing malignant changes and pool exhaustion (13, 14). Certain factors can turn this quiescent state into an active phase, such as tissue signals produced following tissue damage (15, 16). Also, these cells, which are responsible for the regeneration and repair of the tissue and damaged organs, have more ability to withstand various stresses (17). It has been shown that adult stem cells endure harmful stimuli and remain uninjured under unfavorable conditions, which are required for tissue repair (18). Hence, subjecting MSCs to non-physiological stresses could be a suitable strategy to improve their efficacy in tissue repair. MSC stress responses to various environments, including hypoxia (19, 20, 21, 22, 23, 24), hypothermia (25), hyperthermia (17, 26, 27, 28, 29, 30, 31, 32), and serum deprivation (33, 34), were evaluated in many studies. Temperature is an important environmental and medical factor that regulates various cellular processes. It was demonstrated that hyperthermia has a "hormesis' effect on MSCs (28). This means elevated temperature up to a certain threshold has a stimulatory effect on stem cell behaviors. However, in higher quantities, it is

considered lethal and induces senescence and apoptosis (26). Studies showed mild heat stress enhanced MSC differentiation (17, 31, 32, 35, 36). Also, the increase in expression of heat shock proteins (HSPs), molecular chaperones responsible for repairing damaged proteins and assisting in protein translocation, in heat-induced cells has been demonstrated in many studies (17, 26, 27, 37). It was also reported that elevated temperature could enrich heterogeneous stem cell populations, improving their maximum potential for proliferation and differentiation (17).

Although cell responses to heat stress are one of the most examined cellular stress responses, most studies, in this context, investigated the characteristics of MSCs exposed to heat shock stress for a short period (17, 26, 27, 28, 29, 30, 31, 32). The current study aimed to investigate the properties of MSCs, derived from dental pulp stem cells (DPSCs) following long-term heat stress. The advantage of MSCs derived from dental pulp, DPSCs, is their isolation's affordable, painless, and relatively noninvasive manner (38). Cells were cultivated at elevated temperatures up to 39 °C, which has been shown to significantly enhance the proliferation and differentiation of various MSCs (31, 32). Moreover, since *in vivo* regenerative capability of MSCs under heat stress remain unexplored, we also transplanted the stress-endured human DPSCs (hDPSCs) loaded on β-TCP blocks in critical size defects created in a rat calvaria model for bone regenerative purposes.

Materials and Methods

Isolation and culture of hDPSCs

The dental pulp tissue was gently extracted from the crown and root of the removed wisdom tooth of healthy volunteers and immersed in a solution of 0.75% collagenase type 1 for 1 hour at 37 °C. The initial cell suspension was immersed in Dulbecco's Modified Eagle Medium- High glucose (DMEM-HG) and fetal bovine serum (FBS) 10% and 100 units per ml penicillin and 100 mg/ml streptomycin, cultured in 25 cm2 culture flasks, and placed in a humid incubator with 5% CO2 and 37 °C. The cells were cultured and passaged in a confluency of 80 to 90% by 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) enzyme with a ratio of 1 to 3. The second passage cells were maintained for further studies in the standard freezing method. One hundred six cells were transferred to a frozen culture medium containing 10% dimethyl sulfoxide (DMSO) in a 2 ml cryovial. Then they were transferred to liquid nitrogen (196 °C). Cultivation continued until passages 3-5 for subsequent analysis.

Characterization of hDPSCs by flow cytometry

Expression of surface markers, including CD90, CD73, and CD105, and the absence of CD45 and CD34 markers was evaluated using Fluorescence-activated cell sorting (FACS). Monoclonal fluorescein isothiocyanate and phycoerythrin-conjugated antibodies were placed on the isolated cells. A concentration of 2 µg / ml of Anti-CD90-FITC, Anti-CD73-PE, Anti-CD105-PE, Anti-CD45-FITC, and Anti-CD34-PE (EXBIO Praha, Vestec, Czech Republic) were used. The negative control included the cells stained with rat G immunoglobulin labeled with FITC. Labeled cells were evaluated using Flow Cytometry (BD FACS Calibur; BD Biosciences, Franklin Lakes, NJ, USA). FlowJo 7.6.1 software (FlowJo LLC, Ashland, OR, USA) used to analyze the standard flow cytometry files. Samples containing at least 90% fluorescently labeled cells were considered positive samples.

Assessing the osteogenesis ability of hDPSCs using Alizarin red staining.

To confirm osteogenic differentiation ability, the cells were incubated for 14 days in the osteogenic culture medium including Dulbecco's Modified Eagle Medium- Low glucose (DMEM-LG), 10% FBS, 50 µg / ml ascorbate 2-phosphate, 10-8 M dexamethasone, and 10 mM beta-glycerophosphate. To investigate mineralization, Alizarin red staining was used according to a protocol by Carl A. Gregory *et al.,* (39). In summary, the cells were washed with phosphate-buffered saline (PBS) and fixed at room temperature using a 10% volume formaldehyde for 15 minutes. The cells were washed twice with distilled water, and then 250 µl of Alizarin red (40mmol) was added to the wells. Plates were incubated for 20 minutes at room temperature on a vibrating incubator. After aspiration of non-reacting color, the wells were washed 4 times with 1 ml distilled water while the plates were placed in vibrating incubation condition for 5 minutes. An optical microscope saw the stained layer.

Assessing the fluctuation of incubator temperature

Incubator temperature fluctuations were recorded at 37 °C and 39 °C incubators using an electronic thermometer every 5 minutes and for 5 consecutive days.

Scaffold preparation

First, the initial powder components of calcium phosphate-containing amorphous tricalcium phosphate are converted to a ceramic slush form material (with a ratio of 30 g per ml of distilled water), and to produce desired rheological properties,

suitable additives such as colloidal silica, tripolyphosphate, and carboxymethylcellulose were added to it. The polyurethane sponge was cut to the required dimensions. Sponges were thoroughly washed and dried before immersion in a TCP solution to remove any fat or contamination with a detergent liquid. Then the sponges were placed inside the slush to absorb the bio-ceramic slush components into the foam. Afterward, the samples were brought out of the slush, dried at room and oven temperatures, respectively, and then placed under a heat operation in an appropriate cycle to 1200 °C. To cover the scaffolds with gelatin, 10 weight percent gelatin solution was prepared, and the scaffolds were immersed in the solution. The scaffolds were then placed in the freeze dryer at - 80 °C. Gelatin was cross-linked by 1 weight percent glutaraldehyde and dried again after washing with distilled water at - 80 °C.

Seeding cells on the scaffold

5 × 106 cells were seeded on the scaffold during the following process. Cells were placed in 100 µl of stem cell culture medium. Half of the cells were placed at the top of the scaffold by a pipette. After 20 minutes, the scaffold was reversed, and the remaining cells were placed on the other side of the scaffold and incubated for 20 minutes. The incubation time allowed the cells to enter the scaffold's porous structure.

The effect of heat stress on cell proliferation and osteogenic differentiation of mesenchymal cells extracted from dental pulp tissue was assessed at 39 °C.

In vitro evaluation of the effect of heat stress on cell proliferation and adhesion

Cells seeded on the scaffold were incubated for 24 and 72 hours at 37 °C (control group) and 39 °C.

The 3-(4,5-Dimethylthiazol-2-yl)-2, 5 - diphenyltetrazolium bromide (MTT) test was used to evaluate cell proliferation. For MTT, cells were incubated in 0.5 mg/ml of MTT solution in DMEM at 37 °C for 2 hours. The MTT solution was removed, and a buffer containing DMSO was added to dissolve the Formosan crystals. After 5 minutes, optical density was read using the Elisa reader at 570 nm wavelength. Cell adhesion was observed using 4′, 6-diamidino-2-phenylindole (DAPI) staining; the cells were washed 3 times with PBS. Then, DAPI dye solution was added, and the cells were incubated for 5 minutes at room temperature. The DAPI dye was removed, and the cells were washed 3 times with PBS and then observed under a fluorescence microscope.

In vitro evaluation of the effect of heat stress on osteogenic differentiation

To evaluate osteogenic differentiation, cells seeded on scaffolds were cultured at 37 and 39 °C and in an osteogenic culture medium. Alkaline Phosphatase (ALP) activity was evaluated after 7 and 14 days. The cell-containing structures were washed with PBS and then homogenized in the lysis buffer (pH 5.7, 10 mMol-1 Tris-HCL, 1 mM-1 MgCl2, and 0.05% Triton X-100). The resulting mixture was centrifuged at 12000 rpm for 10 minutes at 4 °C. Then, mixed with a P nitro phenol phosphate substrate and alkaline buffer solution. After incubation at 37 °C for 15 minutes, the resulting mixture was combined with 0.5 mmol/L NaOH to stop the reaction. Elisa reader then read the optical absorption at the wavelength of 405 nm.

In vivo evaluation of the effect of heat stress on osteogenic regeneration in an animal model

For *in vivo* evaluation, we used five-month-old Sprague Dawley rats. Before surgery, rats were anesthetized by intramuscular injection of ketamine (100 mg per kg of body weight) and xylazine (10 mg per kg of body weight). Warm water bags were used to prevent decreasing body temperature in rats. The skin hair around the cutting area was removed and disinfected with a 5% betadine solution. One cut was made by a surgical blade from the nasofrontal region to the anterior regions of the occipital protuberance. After that, a full thickness defect, 5 mm in diameter, was made using a low-speed XEMAX Trephine drill behind the coronal suture in each sample, along with sufficient amounts of washing using sterilized saline. Surgery was carried out without damage to the dura mater and its vessels. Cell-scaffold structures were washed three times by PBS to separate the culture medium and then placed in the lesion. 16 rats were randomly categorized in the following groups: Group 1, hDPSC cells cultured at 37 °C on the β-TCP scaffold (N=4); group 2, hDPSC cells cultured at 39 °C on the β-TCP scaffold (N=4), group 3, β-TCP scaffold without cells (N=4) group 4, defects without any treatment as the negative control group (N=4). After transplantation, the periosteum was closed using a 5-0 polyglycolic suture, and the skull skin was closed using a 4-0 nylon suture. After surgery, the animals were placed in a warm plastic cage with a soft bed for recovery. To prevent infection, subcutaneous injection of endrofloxacin 5% was performed for 3 days. Animals were sacrificed after eight weeks, and their skull was removed.

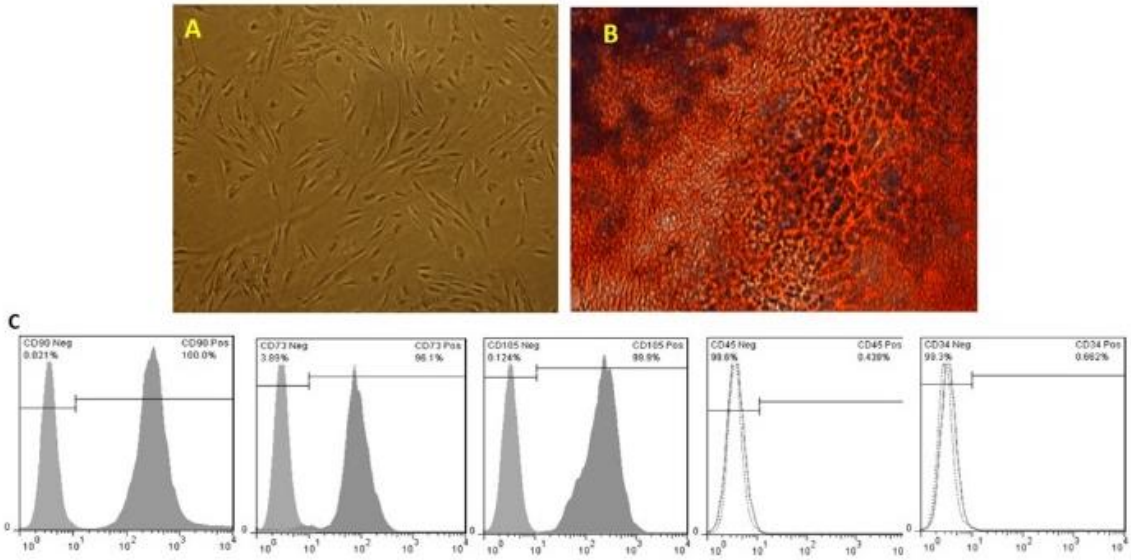

Figure 1. DPSCs characterization. A) DPSCs morphology at passage 2 after 7 days in standard culture medium, B) osteogenic capability of DPSCs after 14days, C) Flow cytometry analysis of DPSCs for mesenchymal stem cell surface markers

Bone samples were placed in a fixative solution containing natural buffered formalin for one week and then decalcified in Cal-Ex Decalcifier for 2 to 3 weeks. Each sample got fixed with paraffin. The 5mm serial cuts were made parallel to the sagittal axis. 20 sections were prepared for each sample and placed on Sperfrost Plus slides. Xylan was used for the isolation of paraffin. The slides were stained with Hematoxylin and eosin. Digital images of stained slides were made by optical microscopy.

Statistical Analysis

IBM SPSS Statistics for Windows (Version 22.0. Armonk, NY: IBM Corp) performed the statistical analysis. Students` T-test was used to analyze differences between *in vitro* groups. We used a univariate general linear model to assess the interaction of time and temperature on cell proliferation and osteogenic differentiation.

Ethical Consideration

The study protocol and all animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) and the Ethics Committee of Shahid Beheshti University of Medical Sciences (code: IR.SBMU.RIDS.REC.1395.254).

Results

hDPSCs characterization

Figure 1A showed the fibroblast-like morphology of hDPSCs cultured in standard culture media for 7 days (Figure 1A). Also, to confirm the osteogenic capability of cells, hDPSCs were cultured in an osteogenic culture medium for 14 days. They were observed using optical microscope after staining with Alizarin red (Figure. 1B).

The stemness nature of hDPSCs cells was evaluated by analysis of MSC surface markers using flow cytometry. According to the results of flow cytometry that are shown in Figure. 1C, the expression of CD90, CD73 and CD105 markers and absence of CD45 and CD34 markers were confirmed.

Temperature fluctuations of incubators

The temperature fluctuations of 37 ° and 39 °C incubators were evaluated using an electronic thermometer, which had been set to record the temperatures every 5 minutes for 5 consecutive days. No changes were not observed in both incubators over the 5 days (Figure 2), and it was concluded that both incubators could keep the temperature of the culture medium stable during the study period.

In vitro evaluation of the effect of heat stress on cell proliferation and adhesion

The proliferation of hDPSCs after 24 and 72 hours of culture in a standard culture medium at 37 °C and 39 °C was evaluated using an MTT assay. After 24 hours, there was no significant difference between the groups (*P* value = 0.828); however, after

72 hours, cell proliferation in the 39 °C group was significantly higher than in the 37 °C group (*P* value = 0.025). Also, in both 37 and 39 °C groups, cell proliferation after 72 hours was significantly higher than 24 hours (*P* value = 0.0001). Assessing the simultaneous effect of time and temperature on cell proliferation showed that the cell proliferation after 72 hours and in the 39 °C group was significantly higher (*P* value = 0.001) (Figure.3).

hDPSC attachment on the scaffolds was also determined at 37 and 39 ° C groups, after 24 and 72 hours, using DAPI staining (Figure.4). As shown, hDPSCs were significantly proliferated faster on scaffold incubated at 39 ° C.

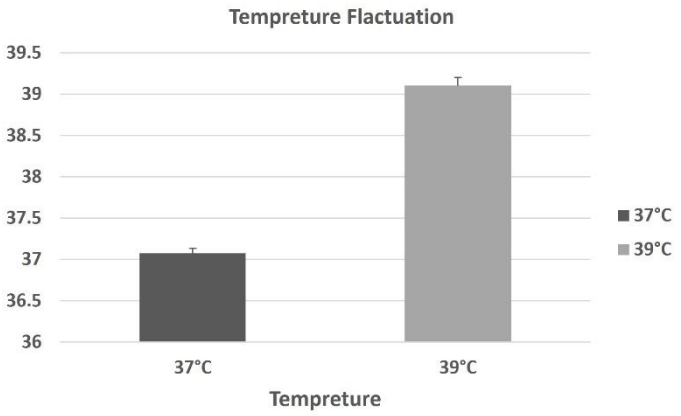

Figure 2. Evaluation of temperature fluctuation at 37 and 39 °C incubation in the periods of 5 days

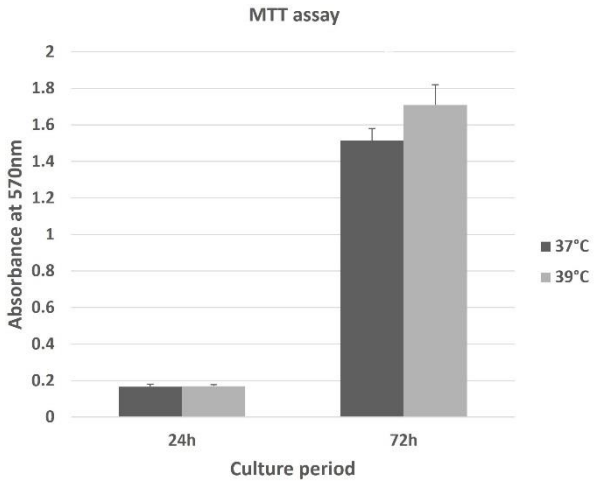

Figure 3. Evaluation of proliferation of DPSC loaded on scaffolds using MTT assay after 24 and 72h after incubation at 37°C and 39 °C

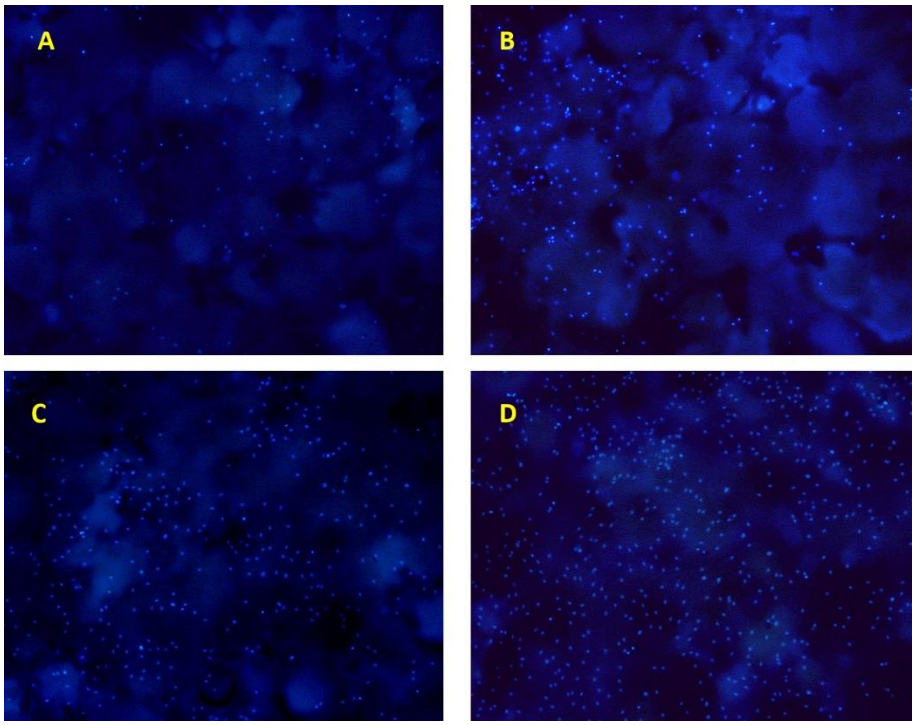

Figure 4. Evaluation of DPSC attachment on scaffold using DAPI staining. cells were incubated at 37°C for 24h (A) and 72h (B) and 39 °C for 24h (C) and 72h (D)

In vitro evaluation of the effect of heat stress on osteogenic differentiation

The osteogenic differentiation of hDPSCs was evaluated after 5 days of culturing in standard culture media (control) and 7 and 14 days in osteogenic culture medium at 37 and 39 °C, using ALP activity assay. After 5 days of culturing in a standard culture medium (*P* value = 0.008), 7 days of culturing in an osteogenic culture medium (*P* value = 0.0001), and 14 days of culturing in an osteogenic culture medium (*P* value = 0.021), the activity of ALP in the 39 ° C group was significantly higher than 37 °C group (Figure 5).

Also, in both 37 and 39 °C groups, ALP activity significantly decreased after 14th day compared to the 7th day (*P* value = 0.009 for the 37 °C group and *P*-value = 0.0001 for the 39 °C group).

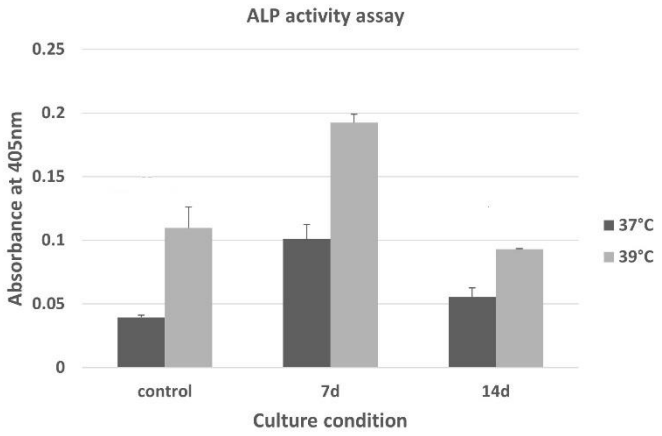

Figure5. Evaluation of DPSC osteogenic differentiation using ALP activity after incubation at 37°C and 39 °C for 5days in standard culture medium (control) and 7and 14 days in osteogenic induction medium

In vivo study of the effect of heat stress on bone regeneration in an animal model

Eight weeks postoperatively, samples were stained with Hematoxylin and eosin (Figure 6). The highest new bone formation was observed in 39 °C. Negative control showed no new bone formation.

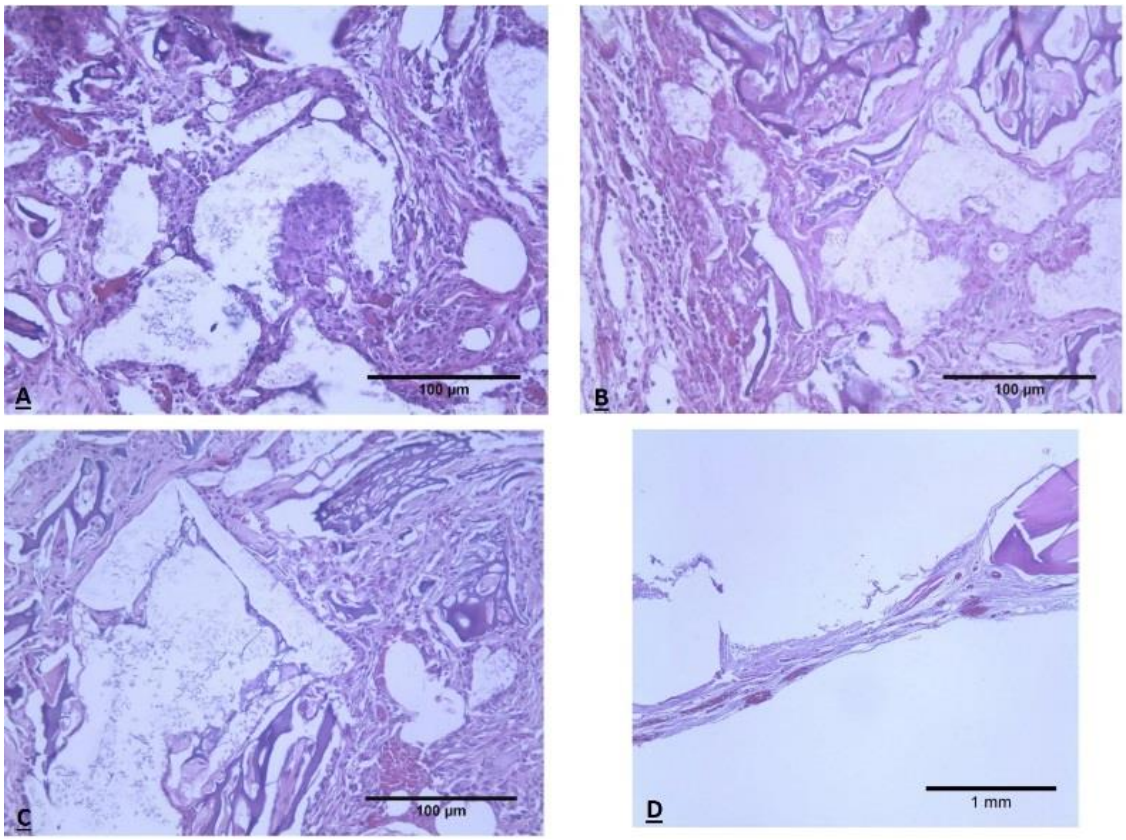

Figure 6. Bone regeneration capability of DPSCs loaded on scaffold incubated in various temperatures after eight weeks in a rat calvaria model using Hematoxylin and eosin staining. A) cell-scaffold incubated at 37°C with 200X magnification, B) cell-scaffold incubated at 39°C with 200X magnification, C) scaffold-only with 200X magnification, and D) no scaffold group with 40X magnification

Discussion

Since MSCs hold great promise for regenerative medicine, various strategies to maintain and improve their plasticity and efficiency are attempted in various biomedical research (40, 41). The application of heat stress on MSCs during *in vitro* cultivation contributed to the fact that in a heterogeneous population of adult stem cells, a stem cell counterpart can endure elevated temperatures more significantly than that non-stem cells, which could be beneficial to purify the MSC population (17). Moreover, previous studies showed mild heat stress enhanced MSC behaviors, including proliferation and differentiation (17, 26, 27, 28, 29, 30, 31, 32). In contrast to previous reports, in which mild heat stresses were administrated in a short period, in this study, we used heat stress not only during cultivation time but also during osteogenic induction. Heat-stressed DPSCs loaded on β-TCP blocks were evaluated for their proliferation and differentiation capabilities *in vitro*. Also, their efficacy for bone regeneration in a rat calvaria model was evaluated for the first time.

It is agreed that heat stress has a hormesis effect on MSC proliferation (42). Previous studies showed that increased temperatures up to 39 and 40° C enhance the proliferation capability of MSCs (17, 31). However, raising the temperature to 41-45° C has been shown to have an inhibitory effect on cell proliferation/viability, ranging from a decrease in cell doubling to inducing cell cycle arrest, premature senescence, and cell apoptosis/necrosis (17, 26, 31, 43). The difference in the severity of the effect on cell proliferation depends on the type of stem cells, duration of heat stress, and, more importantly, the temperature administrated (17, 26, 31, 43). Surprisingly, raising the temperature to 45° C has contradicting results on the age of MSCs (27, 28). Studies by Andreeva *et al.,* and Choudhery *et al.,* showed that heat stress significantly activates the proliferation of aged MSCs, indicating that heat stress may have a certain rejuvenating effect on these populations (27, 28). In the current study, mild heat stress (39 ° C) was administered during cultivation (3-5 days) of young hDPSCs (Passage 3-5). The increased proliferation capability of the cells at 39 ° C was seen, which be well attributed to the hormesis phenomenon.

Heat-endured or heat-stressed MSCs have been shown to have increased multilineage differentiation capabilities (17, 31, 32, 35, 36). The increase in their differentiation capabilities could be related to various explanations. First, several studies showed that MSC cultivation under improper conditions like elevated temperature was exploited to enrich stem cell populations (17, 33, 34). These cells were called multilineage differentiating stress-enduring cells by Kuroda *et al.,* (34). Secondly, these cells have been shown to significantly express differentiation markers (17, 31), indicating the stimulatory effect of elevated temperature on the activation of signaling transduction pathways. Studies by Lee *et al.,* and Yao *et al.,* showed although raising the temperate above 39° C could inhibit proliferation rates dramatically, survival cells showed enhanced differentiation capabilities (17, 29). Despite this stimulatory effect, since the harmful effect of higher temperature on MSC proliferation would eventually reduce the number of transplanted cells, which is clinically relevant, in this study, 39° C was not only used for the cultivation period (5 days in standard medium) but also administrated during osteogenic induction (for 14 days). The results showed that administration of 39° C, for almost 20 days, on hDPSCs, increased their osteogenic differentiation capabilities. The results indicated that both proliferative and differentiation capability of MSCs can be maintained by mild heat shock up to 39° C during *in vitro* expansion and differentiation.

Early studies have shown that the longitudinal and concentric growth of the femur and tibia in animal models could be stimulated by heat (1.5–3.0°C above normal) (44, 45). Also, increasing the temperature has been shown to positively affect body weight, total calcium content, and the length of long bones in chicken embryos (46). Bone resorption and new bone formation have been induced by local hyperthermia (1–4°C above normal) in a rat calvaria model (47) and also, in the femur of rabbits after initial surgical trauma by hyperthermic treatment (42.5–44°C) (48). These studies suggest that the stimulatory effect of hyperthermia on the progeny of osteoblasts accelerates local bone formation. The important concern in the cultivation of stem cell/progenitor cells under heated stress for a lengthy period is the fact that whether in the survived cells, their stemness is compromised or not. To our knowledge, no report is available regarding evaluating the progeny of stem cells subjected to heat stress for bone tissue regeneration purposes. Hence, in this study, stress-induced hDPSCs loaded on β-TCP blocks were transplanted to the critical size defect of a rat model, and the results showed significant bone regeneration.

Conclusion

The results presented in this study showed that mild heat stress for a long period can maintain and improve the proliferative and differentiation potential of MSCs, *in vitro*. Also, based on the result of bone regeneration *in vivo*, this may be a starting point

differentiation in regenerative medicine.

Conflict of Interest: 'None declared'.

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