

## Research Report

## The role of heat shock protein 27 (HSP 27) as inhibitor apoptosis in hypoxic conditions of bone marrow stem cell culture

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### ABSTRACT

**Background:** The concept of stem cell therapy is one of the new hope as a medical therapy on salivary gland defect. However, the lack of viability of the transplanted stem cells survival rate led to the decrease of effectiveness of stem cell therapy. The underlying assumption in the decrease of viability and function of stem cells is an increase of apoptosis incidence. It suggests that the microenvironment in the area of damaged tissues is not conducive to support stem cell viability. One of the microenvironment is the hypoxia condition. Several scientific journals revealed that the administration of hypoxic cell culture can result in stress cells but on the other hand the stress condition of the cells also stimulates heat shock protein 27 (HSP 27) as antiapoptosis through inhibition of caspase 9. **Purpose:** The purpose of this study was to examine the role of heat shock protein 27 as inhibitor apoptosis in hypoxic conditions of bone marrow stem cell culture. **Methods:** Stem cell culture was performed in hypoxic conditions ( $O_2$  1%) and measured the resistance to apoptosis through HSP 27 and caspase 9 expression of bone marrow mesenchymal stem cells by using immunofluorescence and real time PCR. **Results:** The result of study showed that preconditioning hypoxia could inhibit apoptosis through increasing HSP 27 and decreasing level of caspase 9. **Conclusion:** The study suggested that hypoxic precondition could reduce apoptosis by increasing amount of heat shock protein 27 and decreasing caspase 9.

**Key words:** Mesenchymal stem cells, hypoxia, HSP 27, caspase-9, apoptosis

### ABSTRAK

**Latar belakang:** Konsep terapi stem cell merupakan salah satu harapan baru sebagai terapi medis kelainan kelenjar ludah. Namun, rendahnya viabilitas stem cell yang ditransplantasikan menyebabkan penurunan efektivitas terapi. Asumsi yang mendasari rendahnya viabilitas dan fungsi stem cell adalah tingginya kejadian apoptosis. Hal ini menunjukkan bahwa lingkungan mikro di daerah jaringan yang rusak tidak kondusif untuk mendukung viabilitas stem cell. Salah satu lingkungan mikro adalah kondisi hipoksia. Beberapa jurnal ilmiah mengungkapkan bahwa kondisi hipoksia pada kultur sel dapat menyebabkan sel-sel stres, namun di sisi lain kondisi stres sel juga merangsang heat shock protein 27 (HSP 27) sebagai antiapoptosis dengan menghambat ekspresi caspase 9. **Tujuan:** Tujuan penelitian ini adalah untuk meneliti peran protein heat shock 27 sebagai inhibitor apoptosis dalam kondisi hipoksia kultur stem cell sumsum tulang. **Metode:** Kultur stem sel dilakukan dalam kondisi hipoksia ( $O_2$  1%) dan mengukur resistensi terhadap apoptosis melalui ekspresi HSP 27 dan caspase 9 stem cell mesenchymal sumsum tulang dengan menggunakan immunofluorescence dan PCR real time. **Hasil:** Hasil penelitian menunjukkan bahwa prakondisi hipoksia dapat menghambat apoptosis melalui peningkatan HSP 27 dan penurunan tingkat Caspase 9. **Simpulan:** Studi ini menunjukkan bahwa prakondisi hipoksia dapat mengurangi apoptosis dengan meningkatkan jumlah protein heat shock 27 dan penurunan caspase 9.

**Kata kunci:** Mesenchymal stem cells, hypoxia, HSP 27, caspase-9, apoptosis

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## INTRODUCTION

Stem cell transplantation has been explored as a therapy to restore the function of various damaged organs like skin, cornea, heart disease etc, but some of them the probability of success to regenerate tissue using cell transplantation still remains uncertain. The possibility to transplant the cells strongly attaching and surviving in the defect area can integrate with the surrounding microenvironment or the so-called stem cell niche. Niche stem cell is required to improve and maintain the viability of the transplanted stem cells in salivary gland so it has the same environment-microenvironment - as micro-physiological conditions and can support the cells of origin of stem cells can proliferate and differentiate into acinar like cells.

However, the lack of viability in the form of the survival of the transplanted stem cells results in the effectiveness of stem cell therapy reduced. The temporary underlying assumptions of the decline in the viability and function of stem cells is an increase of incidence of apoptosis.<sup>1</sup> The use of stem cells in myocardial infarction case indicated that more than 99% of the stem cells injected into the heart's left ventricle of adult mice resulted in apoptosis at day 4 after injection.<sup>2,3</sup> It suggests that the microenvironment in the damaged tissue is not conducive to support stem cell viability. One of the microenvironments is the hypoxia condition.<sup>4,5</sup>

Hypoxia conditions are an important element of microenvironment but it is often neglected. In the conventional stem cell culture it is done is normoxia conditions (oxygen concentration 21%). However, contrary to the condition of *in vivo* stem cell requires a hypoxic environment between 1-15% depending on the type of stem cell. This shows that *in vitro* culture also requires the same treatment as the *in vitro* physiological environment, so that the success of the therapy can be achieved.<sup>6</sup> Several scientific journals stated that the administration of hypoxic cell culture can result in the stress of cells which can induce apoptosis.<sup>7</sup> The stress condition of the cells, among others, which is triggered by hypoxia also stimulates the release of heat shock protein 27 (HSP 27). HSP 27 is known as antiapoptosis through inhibits cytochrom-c release and caspase-9.<sup>13-148,9</sup> There is an assumption that HSP 27 inhibits apoptosis in hypoxia conditions. The purpose of this study was to examine the role of HSP 27 as an inhibitor of apoptosis in hypoxic conditions of bone marrow stem cell culture. The result of study could be served as an adaptive and survival stem cells for therapy of salivary gland defect due to ionized radiation.

## MATERIAL AND METHODS

The experimental unit in this study was the mesenchymal stem cells (MSCs) taken from the crista illiaca bone marrow

of male New Zealand rabbits at the age of 6 months and done through worthy of Conduct research (Ethical Codes) in the Faculty of Veterinary Medicine, Universitas Airlangga. This research was exploratory laboratory experimental studies conducted *in vitro* on stem cell culture. Bone marrow mesenchymal stem cell were taken from bone crista illiaca New Zealand rabbits were cultured *in vitro* hypoxic conditions (O<sub>2</sub>1%) in the 3rd passage and compared with normoxia condition (O<sub>2</sub> 21%). Then the incidence of apoptosis, the expression of HSP 27 was analyzed. This research was conducted at the Laboratory of Stem Cell-Institute of Tropical Disease (ITD) Universitas Airlangga Surabaya.

Mononuclear cells were separated by centrifugation at 1600 rpm for 10 min at room temperature. The interface was collected and resuspended in phosphate buffered saline (PBS). The cells were suspended in  $\alpha$  Modified Eagle Medium ( $\alpha$  MEM), supplemented with 10% foetal bovine serum and penicillin-streptomycin sulfate. The cells were plated in 10 cm<sup>2</sup> culture dish. The culture was maintained at 37° C in humidified environment containing 5% CO<sub>2</sub>. After 48 h, the non-adherent cells were removed and medium replaced. When 80-90% confluence was reached, adherent cells was trypsinised with 0.02% trypsin (Hyclone, Logan, UT) at 37° C for 5 min. Then divided into hypoxia precondition treatment oxygen tension 1% and normoxia oxygen tension 21%.

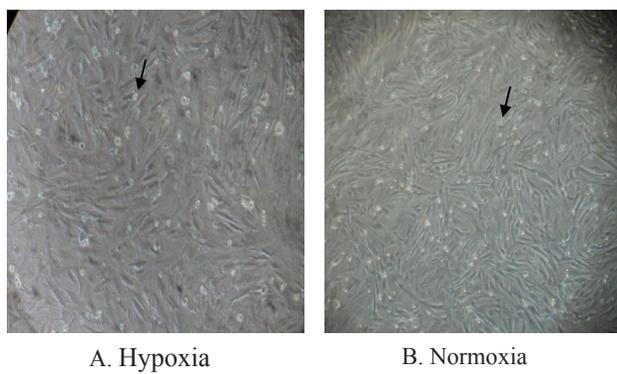
The medium was aspirated and washed with 1 ml PBS 2 times, then add 500  $\mu$ l RNAiso/ well. The cells were scraped and put into the small tube, 100  $\mu$ l Chloroform were added and shook for several times. The supernatant (250-300  $\mu$ l) was separated into a new tube by centrifugation at 12.000 rpm for 15 minutes at 4° C, then added 250  $\mu$ l isopropanol and centrifuge in 12.000 rpm for 10 minutes at 4° C. The supernatant was removed from tube, leaving only RNA pellet, added 500  $\mu$ l 75% ethanol vortex until the pellet floating. The samples were centrifuged at 4° C-12.000 rpm for 5 minutes, the supernatant was removed from tube then dry up for 5-10 minutes, then added 25  $\mu$ l water. The 1  $\mu$ l samples were examined the concentration of RNA for measuring by spectrophotometer and acceptable range for purity of RNA are 1.7–2.1.

The RNA sample was added with water (11  $\mu$ l) and the 9  $\mu$ l mixtures component put into a fresh tube, then mixed with 11  $\mu$ l RNA samples, put it in to thermal cycle machine at 42° C 20 minutes, 99° C 5 minutes, 4° C 5 minutes, 4° C as long as possible. The 20  $\mu$ l c-DNA samples have prepared.

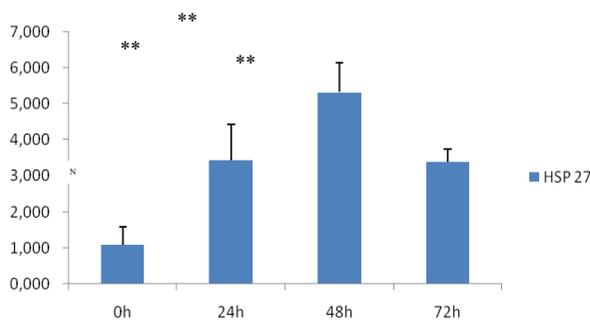
Data obtained are a single type only and calculated quantitatively with numerical data scale. When the distribution is normal, it can be tested by Anova test

**RESULT**

The morphological of MSCs was observed after third passage at 2 days in both cultures of normoxia (O<sub>2</sub> 21%) and hypoxia (O<sub>2</sub> 1%), the cells showed mostly have bigger cells size, less cell death and slower proliferation in hypoxia culture, meanwhile in normoxia culture have smaller cells size, more cell death and faster proliferation rate (Figure 1).

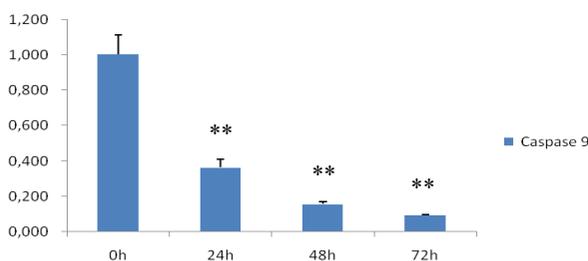


**Figure 1.** The morphological of BMSCs after third passage at 2 days in normoxia with  $\alpha$  MEM + 10% FBS medium's cultures. Cell death (arrow).



\*\*  $p < 0.01$

**Figure 2.** Identification of HSP 27 by using real time-PCR showed significantly increasing HSP 27 expression ( $p < 0.01$ ) in hypoxic precondition (O<sub>2</sub> 1%).



**Figure 3.** Identification of caspase 9 by using RT-PCR showed significantly decreasing of caspase 9 expression ( $p < 0.01$ ) in hypoxic precondition (O<sub>2</sub> 21%).

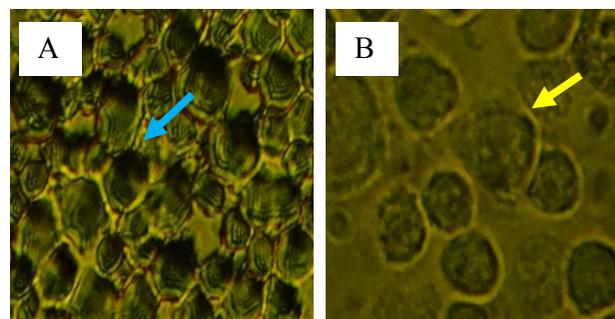
Note: \*\* $p < 0.01$

The identification of heat shock protein 27 and caspase-9 using real time-PCR showed significantly increasing HSP 27 and decreasing caspase-9 expression in hypoxic condition (O<sub>2</sub> 1%) ( $p < 0.01$ ) compared to normoxia condition as a control.

**DISCUSSION**

Hypoxic condition is one of the important elements in the microenvironment of stem cells, because stem cell microenvironment is needed to protect and influence the behavior of stem cells in maintaining the self-renewal capability and viability of the stem cells. In this study on the effect of hypoxia on the incidence of apoptosis through the expression of HSP 27 indicated that the hypoxic precondition had a significant effect on apoptosis resistance. The result of an experiment conducted in a immunocytochemistry showed that in the hypoxic condition (O<sub>2</sub> 1%), cells undergoing apoptosis were only between 2.45-2.55% after the hypoxic precondition was given with the duration for 24 hours, 48 hours, and 72 hours compared to the number of cells cultured under normoxic condition (O<sub>2</sub> 21%) in which the number of cells undergoing apoptosis reached 12.5%.

In addition, the results of morphological examination using by electron microscopy (magnification of 1000x) showed that the hypoxic-preconditioned stem cells had a slower proliferation, a larger cell shape and fewer dead cells, while in the normoxic condition the cell shape was smaller and flat, the proliferation was faster to make the cells confluence resulting in more dead cells. It indicated that the stem cells cultured in vitro also required the same treatment as the condition in vivo, because physiologically stem cells require a lower oxygen concentration (hypoxia) to maintain the viability and the ability to selfrenewal, depending on the type and location of the cells. The results was accordance with the study reported that the



**Figure 5.** Identification of apoptosis evidence using immunocytochemistry in normoxia and precondition hypoxia. (A) Positive expression of apoptosis evidence in normoxia (arrow), number of apoptosis reached 12.5%; (B). Hipoxia precondition 24 h, 48 h, 72 h, showed negative expression of apoptosis (arrow), have a small number of apoptosis cells (2.45-2.55%).

hypoxic precondition of MSCs cultured could maintain the viability and the proliferation rate and had the better ability of self-renewal.<sup>10</sup> Physiologically stem cells required O<sub>2</sub> concentration between 1-7%, hypoxic condition is needed by the stem cells to maintain their plasticity or differentiation ability.<sup>5,6</sup>

The result of an examination on the effect of hypoxia on the HSP 27 with the use of real time PCR indicated that hypoxic precondition affect the increase of the expression of HSP 27 at 24 hours, 48 hours, and 72 hours after the treatment compared to normoxic condition. It indicates that the treatment of hypoxia in cultured cells results in stress cells stimulating the release of HSP 27 as an anti-apoptosis. It also explains how hypoxia influences the decline in the incidence of apoptosis in the stem cells culture through the stimulation of the release of heat shock protein 27 as an antiapoptosis through inhibition of cytochrom-c and caspase 9 release. It is proven by the significant reduction of caspase 9 at 24 hours, 48 hours, and 72 hours after hypoxic precondition but in normoxic condition it increases. These results are consistent with the study resulted that the treatment of hypoxia preconditions increased stress cells, induced apoptosis and stimulating the release of heat shock protein, acting as an antiapoptosis through the barriers of cytochrom-c.<sup>7,8,9</sup> So it can be stated that HSP 27 may act as an inhibitor of apoptosis in hypoxic conditions of cultured cells, generally in vivo HSP 27 acts as a thermo tolerance, cytoprotection and survival cells under stress conditions.<sup>11-13</sup>

The study suggested that hypoxic precondition could reduce apoptosis by increasing amount of heat shock protein 27 and decreasing capase 9. So it can strongly be attached, survive and integrate into the microenvironment of the original cells to achieve the success of therapy.

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#### ACKNOWLEDGEMENT

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